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Estrogen and the dietary phytoestrogen tesveratrol as regulators of the Rho GTPase Rac in breast cancer research

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14. ABSTRACT

Our hypothesis is that estrogen (E2) and low concentrations of resveratrol promote breast cancer invasion and metastasis while high concentrations of resveratrol prevent breast cancer metastasis via regulation of the signaling protein Rac. As proposed in Specific Aim 1, we have shown that dependent on estrogen receptor (ER) status, E2 and resveratrol have differential effects on Rac activity, cell migration/invasion, and cell growth. In the ER beta +ve MDA-MB-231 breast cancer cell line, 5 μM resveratrol, a low physiological concentration, decreased cell proliferation, caused cell cycle arrest at G2/S phase but increased migration and invasion as well as Rac activity. In the ER –ve MDA-MB-435 breast cancer cell line, 5 μM resveratrol did not affect cell growth and cell cycle progression but increased cell migration/invasion. As proposed in Specific Aim 2, we tested the effect of different resveratrol concentrations on mammary tumor growth and metastasis. Our results show that at all concentrations tested, resveratrol increased mammary tumor growth and metastasis. Interestingly, tumors from mice that received resveratrol diets (0.5, 5, and 50mg/kg body weight) exhibited enhanced Rac activity. However, tumor growth or migration/invasion of this cell line could not be inh bited by a commercially available Rac inhibitor NSC-23766. Therefore, we are developing novel Rac inh bitors as anti breast cancer metastasis compounds (Hernandez, et al., submitted). Even though resveratrol alone is limited in use as a breast cancer metastasis preventive, resveratrol in combination with other grape polyphenols reduced breast cancer growth and metastasis to bone and liver (Castillo-Pichardo et al., 2009).

15. SUBJECT TERMS
Estrogen, resveratrol, breast cancer, metastasis, Rac

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Introduction

The focus of this research project is the role of the hormone estrogen (E_2) and the structurally similar natural compound resveratrol on breast cancer invasion and metastasis. E_2 is important for initiation and progression of breast cancers (1;2;2). Increased ERα levels are associated with 50-80% of breast tumors and inhibition of ERα has become a major strategy for prevention and treatment of breast cancer (3-6). During breast cancer progression, cancer cells become resistant to antiestrogen therapy because malignant breast cancers express only ER□ or no ER and overexpress EGFR isoforms EGFR1 or Her-2 (7). Therefore, it is important to test alternative therapies that can be used effectively to treat ER (-) breast cancer.

Resveratrol is a natural compound from grapes and peanuts that is structurally similar to E_2 and interacts with both ERα and ERβ (8-11). Resveratrol has proapoptotic, antigrowth, anti-inflammmatory, antiangiogenic, and anti-invasive properties that makes it an attractive anticancer compound (12-14). Much of the data on potential anticancer properties of resveratrol has been shown *in vitro* with high concentrations of resveratrol ranging from 30 -200 μM (15-21). We and others have shown that resveratrol at 50 μM can inhibit cell migration and invasion (22-25). Resveratrol can exert biphasic effects where low concentrations are estrogenic while high concentrations are antiestrogenic (16;18;26;27). However, the effects of resveratrol in ER□(-) or ER□□ (-) breast cancers are not well understood. Therefore, the ***purpose*** of this study is to investigate the effects of resveratrol on breast cancer progression in metastatic breast cancers that have lost□ER□ and others have demonstrated that activity of the Rho GTPase Rac is necessary for breast cancer invasion and metastasis (28;29). Our published data demonstrate that the effects of E_2 and resveratrol on cell functions relevant for metastasis such as actin cytoskeletal rearrangement to form motile structures, cell migration, and invasion may be mediated by the action Rac (30). Therefore, we formulated the ***hypothesis*** that high concentrations of resveratrol prevent breast cancer invasion and metastasis while E_2 and low concentrations of resveratrol promote breast cancer invasion and metastasis via Rac-regulated mechanisms. Our ***objective*** is to analyze the effect of varying concentrations of E_2, resveratrol, or a Rac inhibitor on breast cancer invasion and metastasis using human metastatic breast cancer cell lines with no ERα expression.

Body

There was a hiatus of funding of this award from May-November 2009 because the PI moved institutions from Universidad Central del Caribe, Bayamon, PR to University of Puerto Rico, Medical Sciences Campus, San Juan, PR. Therefore, the 2009 funds were not awarded until November, 2009 when the PI hired a new postdoctoral fellow (Angelica Santos, Ph.D., start date November 15, 2009) and resumed studies on this proposal. Therefore, we have requested and received a no-cost extension and will complete the project in August 2011.

Since funding of this award, the origin of the MDA-MB-435 cell line has been called into question. It may be of melanoma origin and not breast cancer; however, since we proposed to use this cell line and have 100% success with obtaining mammary fat pad tumors and metastases with this cell line, we continued to use both MDA-MB-231 and MDA-MB-435 for this investigation.

Task 1. Determine the effect of estrogen and resveratrol on metastatic breast cancer cell lines in vitro *(Months 1-24)*

The Table below summarizes our findings on the effects of resveratrol on cell functions relevant for breast cancer metastasis using the two cell lines that were proposed, i.e. MDA-MB-231 (ERα –ve, ERβ +ve low metastatic breast cancer cells) and MDA-MB-435 (ER-ve highly metastatic cancer cells).

Cell Function	MDA-MB-231	MDA-MB-435
Cell proliferation	Decreased by 5 (60%) and 20 μM (99%) resveratrol	No effect with 5 μM resveratrol. Decreased 80% by 20 μM resveratrol
Cell cycle progression	G2/S phase arrest by 5 and 20 μM resveratrol	No change in cell cycle stage
Cell migration/invasion	Increased by 0.5 (90%) and 5 (50%) μM resveratrol. Inhibited 30% by 50 μM resveratrol.	Increased 200% by 5 and 50% by 25 μM resveratrol. No effect with 50 μM resveratrol.
Mammary tumor growth	In progress	Mammary tumors increased 2-3-fold by treatment with 0.5, 5, 10, and 50 mg/kg BW resveratrol.
Metastasis	In progress	Lung and liver metastases increased by 2.5-3-fold with 0.5, 5, 10, and 50 mg/kg BW resveratrol

When we analyzed the effect of resveratrol on the ER –ve breast cancer cell line MDA-MB-435, we found that even though resveratrol had no effect on cell growth or proliferation, low concentrations of resveratrol dramatically increased cell migration (Figure 1). We have reported that similar to estrogen, 5 μM resveratrol increases while 50 μM resveratrol inhibits cell migration of MDA-MB-231 cells (22, 30, 32). We found that estrogen had no effect on MB-435 cell migration/invasion while 5 and 25 μM resveratrol increased cell migration indicating that this effect is ER independent.

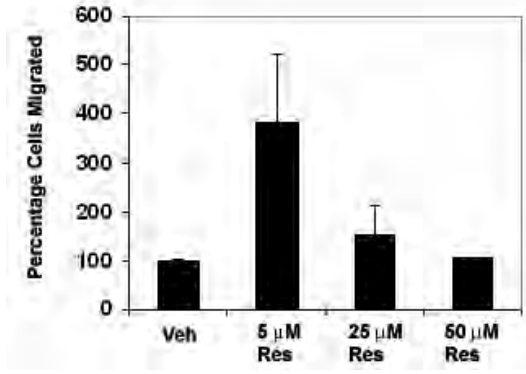

Fig. 1. Effect of resveratrol on MDA-MB-435 cell migration. Quiescent MDA-MB-435 cells were placed on the top well of Transwell chambers in serum-free, phenol red-free media and the number of cells that migrated through the membrane of the top well in response to various treatments was quantified relative to control. Data (±SD) are quantified from analysis of 15 microscopic fields/treatment (n=3). The bottom well contained the following for 12 hours: vehicle, 5, 25, or 50 μM resveratrol.

Similar to the effect of resveratrol in MDA-B-231 cells, where resveratrol at low concentrations (5 μM) increased cell migration, resveratrol dramatically increased migration of the ER-ve MDA-MB-435 cell line. To determine whether the increased breast cancer cell migration/invasion response to resveratrol can affect metastasis in vivo, we next investigated the effect of dietary resveratrol on nude mice with mammary tumors created from the MDA-MB-435 cell line.

Tasks 2 and 3. Determine the effect of estrogen and resveratrol on breast cancer progression (Months 25-36)

To investigate the effect of dietary resveratrol on MDA-MB-435 ER (-) high metastatic cell line in vivo, we established primary tumors by inoculating $5X10^5$ GFP-MDA-MB-435 cells at the mammary fat pad of female athymic nude mice. One week following tumor cell inoculation, mice were gavaged with vehicle (90% neobee oil, 10% ethanol), 0.5, 5, or 50 mg/kg body weight (BW) resveratrol 5 days/week.

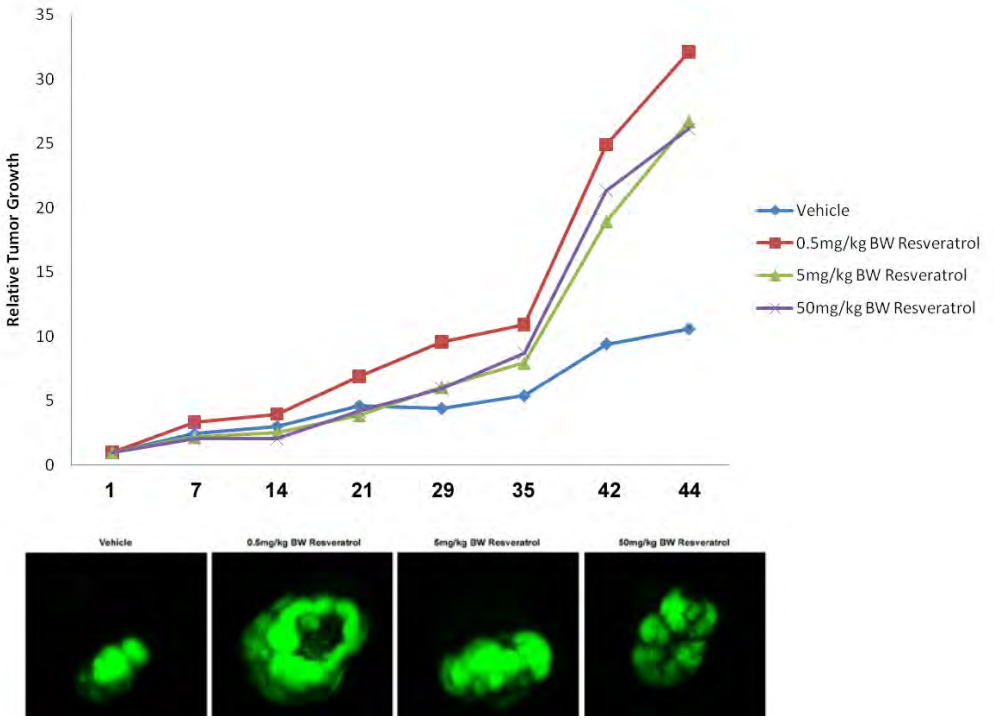

Figure 2. Mammary tumor growth in response to dietary resveratrol. Relative GFP-MDA-MB-435 mammary tumor progression was determined by in situ whole body fluorescence image analysis from day 1 of tumor plantation and compared with images acquired 2X a week for 44 days. Micrographs show representative tumors at day 44 from mice that received vehicle or resveratrol at 0.5, 5, or 50 mg/kg BW.

Tumor growth was monitored by whole body fluorescence image analysis of the fluorescently tagged tumors. Average relative tumor area was calculated from integrated fluorescence intensity of mammary tumors from vehicle or resveratrol at 0.5, 5, or 50 mg/kg BW as described in (31, 32, see Appendix, Castillo-Pichardo, et al., 2009) from 10 mice/group. As shown in Figure 2, all concentrations of resveratrol increased primary mammary tumor growth, in a linear fashion especially after 35 days of dietary administration. Oral treatment of 0.5 mg/kg BW increased mammary tumor growth by 3 -fold and 5 and 50 mg/kg BW resveratrol increased mammary tumor growth by 2-fold compared to vehicle on day 44 (Fig. 3).

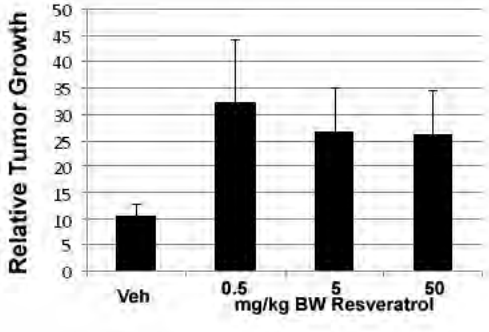

Figure 3. Relative GFP-MDA-MB-435 mammary tumor growth on day of sacrifice (d44). Relative GFP-MDA-MB-435 mammary tumor progression was determined by in situ whole body fluorescence image analysis from day 1 of tumor implantation and compared with images acquired 1X a week for 44 days. Average relative tumor area as calculated from integrated fluorescence intensity of mammary tumors from vehicle (Veh) or 0.5, 5, or 50 mg/kg BW resveratrol (Res)-treated (10 mice/group) nude mice.

The mice were sacrificed on day 44 due to high tumor burden of the mice receiving low dietary resveratrol. Following sacrifice, the distant organs were harvested, stored in liquid nitrogen and imaged for fluorescent metastatic foci using a fluorescence stereoscope. The methodology for metastasis analysis was developed using funds from this award and is described in detail in (32, Castillo-Picardo et al., 2009, see Appendix). Our results show that resveratrol treatment at all concentrations tested increased lung (Fig. 4), kidney, and liver metastases by ~3-fold.

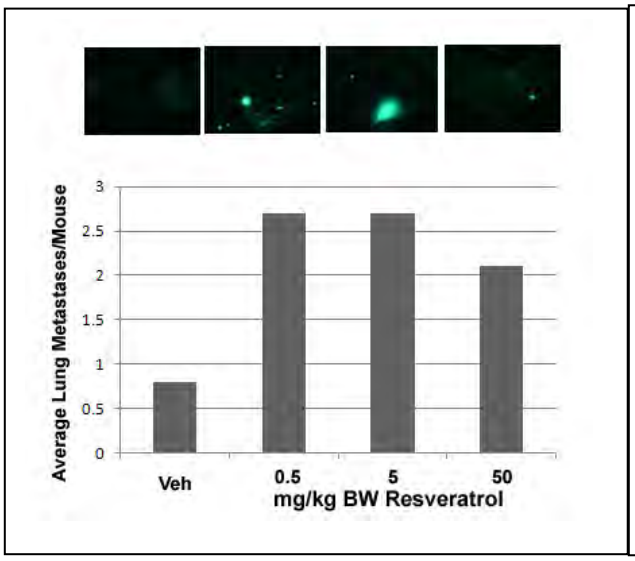

Figure 4. Analysis of lung metastases from mice following resveratrol. Mice bearing GFP-MDA-MB-435 mammary tumors were sacrificed following 44 days of dietary vehicle or resveratrol. The lungs were excised and imaged for green fluorescent metastatic foci in a stereoscope. Representative fluorescence micrographs are shown for each treatment. The average number of lung metastasis/mouse (N=10) was quantified from fluorescent micrographs.

To investigate a function for the small GTPase Rac in the observed increase in mammary tumor growth and metastasis, we excised the tumors at the end of the study and stored them immediately in liquid nitrogen. Tumor extracts were made in lysis buffer as described by us in (33). Rac activity of the tumor lysates was determined by pulldown assays using a GST-fusion protein to the Rac and Cdc42 interactive binding domain of a Rac downstream effector PAK as described in (28, 30). Total Rac and active GTP bound Rac were detected by western blotting the Rac1 from total tumor lysates or pulldowns from 3 vehicle treated tumors or 3/group of resveratrol-treated tumors. Resveratrol-treated tumors did not show a change in total Rac expression but demonstrated statistically significant ~1.5-fold increases in Rac activity (Fig. 5).

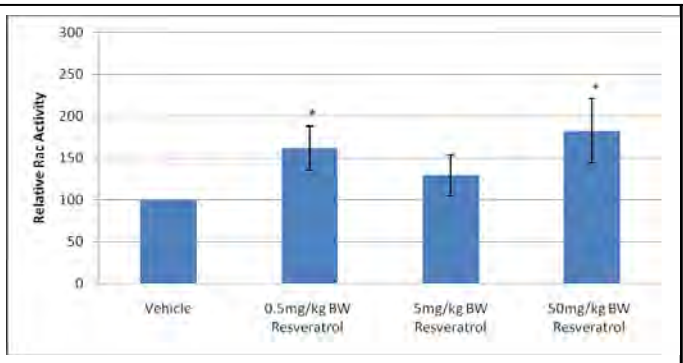

Figure 5. Analysis of Rac activity from GFP-MDA-MB-435 mouse mammary tumors following resveratrol. Mice were sacrificed following 44 days of dietary vehicle or resveratrol. The primary tumors were excised and tumor lysates subjected to Rac activity assays. The relative Rac activity (N=3) as calculated from the integrated density of positive bands in western blots of active Rac1/totalRac1 as a function of the Rac activity of vehicle-treated tumors (100%) is shown.

This intriguing data supports our hypothesis that resveratrol-induced Rac activity can lead to increased breast cancer cell migration/invasion and breast cancer progression to metastasis.

Key Research Accomplishments

Specific Aim 1: In vitro analysis of resveratrol effects on cell functions important for breast cancer metastasis have been completed (Table 1). We have shown that resveratrol can reduce cell proliferation and block cell cycle progression in ERβ positive breast cancer cells but not ER negative breast cancer cells. ER-ve breast cancer cell proliferation and cell cycle progression is not affected by resveratrol treatment. Resveratrol increased cell migration/invasion of both breast cancer cell lines.

We are currently investigating the Rac upstream effectors that may be under resveratrol regulation.

Specific Aim 2: In vivo analysis of resveratrol and Rac inhibitor (NSC-23766) effects on breast cancer metastasis has been completed using the ER (-) MDA-MB-435 metastatic cancer cell line. Our interesting data show that resveratrol increases mammary tumor growth and metastasis of MDA-MB-435 cells. Estrogen or the commercially available Rac inhibitor did not affect MDA-MB-435 mammary tumor growth or metastasis. However, our hypothesis that Rac activity is important for resveratrol-mediated effects on breast cancer is supported by the increased Rac activity of breast cancer cells and mammary tumors following dietary resveratrol.

We have initiated a SCID mouse study (start date May 31, 2010, expected end date August 31, 2010) to analyze the effect of dietary resveratrol on mammary tumor growth and metastasis of ERα (-) ERβ (+) MDA-MB-231 breast cancer cell line.

Since the existing Rac inhibitor NSC-23766 did not inhibit breast cancer cell migration or mammary tumor growth, we are developing novel more efficient Rac inhibitors. We have characterized the inhibitory effects of potential candidate EHop-023 in vitro and are currently assessing its effects in vivo.

Reportable Outcomes

- Because resveratrol alone may not be a viable candidate as a breast cancer metastasis preventive, we tested the effect of a combination of grape polyphenols resveratrol, quercetin, and catechin in vitro and in vivo. This study demonstrated that combined grape polyphenols were viable alternatives to resveratrol alone as breast cancer metastasis preventives and this is published in:

1. Castillo-Pichardo L, Martínez-Montemayor MM, Martínez JE, Wall KM, Cubano LA, **Dharmawardhane S.** Inhibition of metastases to bone and liver by dietary grape polyphenols. Clin Exp Metastasis. 2009;26:505-16 (see manuscript in Appendix).

- The study of the role of resveratrol alone and in combination with quercetin and catechin on breast cancer metastasis resulted in the following poster presentations (abstracts) in 2008-2009 at international conferences:

1. Castillo-Pichardo, L., De La Mota-Peynado, A., Maritnez-Montemayor, M., Otero-Franqui, E., and **Dharmawardhane, S.** Inhibitory mechanisms of breast cancer cell and tumor growth by red wine polyphenols. 100th Annual Meeting of the American Association of Cancer Research. Denver, CO, April 18-22, 2009.

2. Castillo-Pichardo, L., and **Dharmawardhane, S.** Role of estrogen receptor isoform expression on inhibition of breast cancer progression by grape polyphenols. 48th Annual meeting of the American Society of Cell Biology, San Francisco, CA, December 113-17, 2008.

3. Dharmawardhane, S, Azios, N G, Castillo-Pichardo, L, and De La Mota-Peynado, A. Estrogen and resveratrol as regulators of the Rho GTPase Rac in breast cancer metastasis Era of Hope, Deaprtment of Defense Breast Cancer Research Program Meeting, Baltimore, MD, June 25-27, 2008.

- Since NSC32766 the commercially available Rac inhibitor did not inhibit Rac activity, breast cancer cell migration/invasion, or metastasis in the breast cancer cell lines tested, we are developing novel NSC-23766 analogs that are more efficient than the parent compound. Preliminary data from this study was submitted for publication (see Appendix):

1. Hernandez, E, De La Mota, A, **Dharmawardhane, S**, Vlaar, C. Novel Inhibitors of Rac1 as Anti Breast Cancer Metastasis Compounds. PRHSJ, submitted.

- Using preliminary data from this study, funding was requested from NIH/NCI to investigate a breast cancer metastasis inhibitory role for combined grape polyphenols resveratrol, quercetin, and catechin.

1R01CA151718-01
Date of Submission: October 02, 2009
Title: "Grape polyphenols in bone metastasis: potential for combinatorial therapy
Status: Scored, Pending council review, percentile, 40%
Will resubmit in November 2010.

Conclusions

The proposed ***hypothesis*** that high concentrations of resveratrol prevent breast cancer invasion and metastasis while E_2 and low concentrations of resveratrol promote breast cancer invasion and metastasis via Rac-regulated mechanisms was validated, but shown to be dependent on ER expression. Our results show that low concentrations of resveratrol act similar to estrogen and increases Rac activity and cell migration/invasion while high concentrations inhibit Rac activity, Rac/Rac.GEF association, and cell migration/invasion of ERβ (+) metastatic breast cancer cells. However, in ER (-) MDA-MB-435 cells, estrogen had no effect and resveratrol at all concentration tested increased Rac activity and cell migration/invasion. Resveratrol also reduced cell proliferation and inhibited cell cycle progression at S-phase in MDA-MB-231 cells but this inhibitory effect was not as pronounced in MDA-MB-435 cells.

Interestingly, in both MDA-MB-231 and MDA-MB-435 metastatic cancer cells, a combination of resveratrol, quercetin, and catechin at 0.5 and 5μM significantly decreased cell proliferation and induced cell cycle arrest and apoptosis. Therefore, we tested the effect of combined grape polyphenols on tumor growth at the mammary fatpad and metastasis using the highly metastatic ER (-) cell line MDA-MB-435. As shown by our previous studies using MDA-MB-231 cells (30), this study with MDA-MB-435 cells showed that combined grape polyphenols at 5mg/kg body weight each resveratrol, quercetin, and catechin reduced mammary tumor growth. We also recently reported that combined resveratrol, quercetin, and catechin can specifically inhibit metastasis to bone and liver (31), see Appendix.

We also developed novel Rac inhibitors that were more efficient than the commercially available NSC-23766 Rac inhibitor that we intended to use in the orginal proposal (Hernandez, et al., submitted, see Appendix). However, this inhibitor was not sufficient to inhibit all of the Rac activity of the breast cancer cell lines with high endogenous Rac activity. Therefore, we developed and identified a NSC-23766 derivative EHop-023 that can be used for the proposed experiments.

The PI moved her laboratory in July 2009 to the nearby University of Puerto Rico, Medical Sciences Campus (UPR-MSC). Therefore, the funds for 2009-2010 were not received until November 2009. We have submitted and received approval for the animal protocol at UPR-MSC. We recently completed an in vivo investigation of the effect of dietary resveratrol on nude mice with GFP-MDA-MB-435 tumors. We found that resveratrol at all concentrations tested (0.5, 5, 50 mg/kg BW) increased mammary tumor growth and metastasis to lung, liver, kidneys, etc. compared to vehicle alone. The mammary tumors from mice that received resveratrol diets also showed enhanced Rac activity compared to controls indicating that Rac activity may regulate increased breast cancer progression in response to resveratrol. These intriguing data show that dietary concentrations of resveratrol may increase breast cancer growth and metastasis in a Rac-dependent and ER independent manner.

We have requested and received a no-cost extension for this award. We have initiated an investigation to determine the effect of dietary resveratrol on immunocompromised mice with MDA-MB-231 mammary tumors. The mice have been inoculated with GFP-MDA-MB-231 cells at the mammary fat pad and will receive vehicle, 0.5 mg/kg BW 17β-estradiol, 5 or 50 mg/kg BW resveratrol, 10 mg/kg BW NSC23766 or EHop-023. This study is expected to be completed in August/September 2010. To delineate the molecular mechanisms of resveratrol action in ER (-) and ERβ (+) breast cancer, we will excise mouse mammary tumors at the end of these studies and perform PCR arrays and western blotting for genes and proteins associated with Rac signaling and invasion/metastasis.

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RESEARCH PAPER

Inhibition of mammary tumor growth and metastases to bone and liver by dietary grape polyphenols

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Abstract The cancer preventive properties of grape products such as red wine have been attributed to polyphenols enriched in red wine. However, much of the studies on cancer preventive mechanisms of grape polyphenols have been conducted with individual compounds at concentrations too high to be achieved via dietary consumption. We recently reported that combined grape polyphenols at physiologically relevant concentrations are more effective than individual compounds at inhibition of ERα(−), ERβ(+) MDA-MB-231 breast cancer cell proliferation, cell cycle progression, and primary mammary tumor growth (Schlachterman et al., Transl Oncol 1:19 27, 2008). Herein, we show that combined grape polyphenols induce apoptosis and are more effective than individual resveratrol, quercetin, or catechin at inhibition of cell proliferation, cell cycle progression, and cell migration in the highly metastatic ER (−) MDA-MB-435 cell line. The combined effect of dietary grape polyphenols (5 mg/kg each resveratrol, quercetin, and catechin) was tested on progression of mammary tumors in nude mice created from green fluorescent protein-tagged MDA-MB-435 bone metastatic variant. Fluorescence image analysis of primary tumor growth demonstrated a statistically significant decrease in tumor area by dietary grape polyphenols. Molecular analysis of excised tumors demonstrated that reduced mammary tumor growth may be due to upregulation of FOXO1 (forkhead box O1) and NFKBIA (IκBα), thus activating apoptosis and potentially inhibiting NfκB (nuclear factor κB) activity. Image analysis of distant organs for metastases demonstrated that grape polyphenols reduced metastasis especially to liver and bone. Overall, these results indicate that combined dietary grape polyphenols are effective at inhibition of mammary tumor growth and site-specific metastasis.

Keywords Breast cancer · Catechin · Metastasis · Quercetin · Resveratrol

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Introduction

Breast cancer is the most commonly diagnosed form of cancer and the second major cause of death from cancer in women [1, 2]. Recent clinical advances have remarkably increased the survival rates from primary breast cancer; however, the prognosis of breast cancer patients is still limited by metastases that can occur years after initial diagnosis and potential cure. Malignant breast cancers often overexpress epidermal growth factor receptor (EGFR) isoforms such as Her-2 that further confound effective treatment of metastatic breast cancer [3]. Therefore, investigation of the effect of dietary alternatives and their mechanisms of action specifically on Her-2 overexpressing metastatic cancers can lead to alternative therapeutic strategies.

Grape skins and thus red wine, contain many polyphenols that have anticancer properties [4, 5]. Grape polyphenols have been implicated in cancer protection in numerous in vitro studies due to antioxidant and pro-apoptotic effects as well as inhibition of a number of tumorigenic pathways

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[6 8]. Combined grape polyphenols extracted from red wine have been shown to specifically inhibit the growth of breast cancer cells with low cytotoxicity towards normal mammary epithelial cells [9]. However, the effects of grape polyphenols on metastatic breast cancer remain to be investigated.

Resveratrol, quercetin, and catechin, grape polyphenols selected for this study, represent about 70% of the total polyphenols in red wine and have been shown to be the most effective anticancer compounds in red wine [8, 10]. Resveratrol is found in low, but significant amounts in red wine and comprises about 1% of total polyphenols [10, 11]. In breast cancer, resveratrol has been implicated in prevention of multistage carcinogenesis [12, 13]. Quercetin comprises about 6% of total polyphenols in red wine [10] and has been reported to decrease Her-2 expression [14]. Her-2 is often overexpressed in metastatic cancers including the MDA-MB-435 cell line that was used for this study. The monomeric form of catechin constitutes up to 65 70% of total red wine polyphenols and has been shown to delay tumor initiation [10, 15, 16]. Resveratrol, quercetin, and catechin are all viable chemopreventives because they are absorbed and metabolized rapidly in vivo and can be detected in plasma and urine samples in the intact form in humans and rodent models [17 20].

Individually, resveratrol, quercetin, or catechin induce cell cycle arrest and apoptosis in cancer cells [21 23], prevent breast carcinogenesis and cancer progression in rodent models [24 26], and inhibit angiogenesis [27]. Much of the data on the cancer preventive effects of grape polyphenols have been generated from estrogen receptor (ER) (+) tissue culture cell lines and rodent models using pharmacological concentrations of individual polyphenols [16, 24 26, 28, 29]. We previously reported that in ERα(−), ERβ(+) MDA-MB-231 breast cancer cells, resveratrol is inhibitory at high pharmacological concentrations and acts similar to estrogen by increasing cell functions and signaling relevant for metastasis at low dietary levels [30, 31]. However, the effects of combined grape polyphenols at low, dietary concentrations are only now beginning to be assessed.

Recently, we reported that combined resveratrol, quercetin, and catechin (RQC) treatment at physiologically relevant concentrations was more efficient than individual grape polyphenols at inhibition of cell proliferation, cell cycle progression, and primary mammary tumor growth of MDA-MB-231 cells [1]. In this study, we were limited in investigation of the role of grape polyphenols as potential metastasis preventives because the low metastatic MDA-MB-231 cell line formed only a few lung metastases. Most breast cancers preferentially metastasize to bone and liver, where ~80% of patients with advanced breast cancer develop bone cancer, causing severe morbidity and mortality [32]. Therefore, for the current study, we selected a bone metastatic variant of the highly metastatic cancer cell line, MDA-MB-435 [33], to test the effect of grape polyphenols on cell proliferation, cell cycle progression, apoptosis, cell migration, tumor growth, and metastatic progression. Mammary fat pad tumors were established in nude mice and we show that dietary grape polyphenols inhibit both primary tumor growth and metastatic cancer progression from the breast to bone and liver. Our results show that this inhibition may be due to upregulation of caspase 3 activity and expression of FOXO1 transcription factor and NFKBIA; molecules known to regulate cancer progression [34 36].

Materials and methods

Cell culture

Human metastatic cancer cell lines MDA-MB-231 (ERα−, ERβ+) (American Type Culture Collection, Manassas, VA, USA) and a bone metastatic variant of MDA-MB-435 (ER−) stably expressing GFP were used for the study (kind gift of Dr. Danny Welch, The University of Alabama at Birmingham, AL, USA) [37]. Cells were cultured in DMEM with 10% heat-inactivated FBS as described in [1, 33].

Cell proliferation and cell cycle progression

MDA-MB-435 (2×10^5) cells in 5% charcoal-stripped FBS were treated every 48 h for 96 h with vehicle (0.2 0.5% DMSO), 0.5, 5, or 20 μM resveratrol, quercetin, or catechin or a combination RQC at 0.5, 5, or 20 μM each. Cells were fixed, nuclei stained with PI and cell proliferation quantified as the number of cells with intact nuclei. Cell cycle stage of MDA-MB-435 cells was determined by flow cytometry of PI-stained cells as previously described in [1], following treatment with 5 μM resveratrol, quercetin, or catechin or 5 μM RQC every 48 h for 96 h.

Caspase 3 activity assay

Apoptosis was analyzed by the caspase 3 activity of cell lysates following vehicle (0.2% DMSO) or 0.5 or 5 μM RQC for 48 h using a Caspase-3 Colorimetric Assay Kit as per manufacturer's instructions (Sigma Aldrich, St Louis, MO, USA). Briefly, the *p*-nitroaniline (pNA) moiety resulting from hydrolysis of acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) by caspase 3 activity was detected at 405 nm ($\varepsilon_{mM} = 10.5$) after incubating the reaction mixture at 37°C for 22 h. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm using a calibration curve prepared with pNA standards. Concentration of pNA was

further converted to caspase 3 activity in μmol of pNA min^{-1} ml^{-1}.

Annexin V staining

Apoptotic cells were detected by fluorescence microscopy of Annexin V-Cy3-18 stained cells as per manufacturer's instructions (Sigma Aldrich, St Louis, MO, USA). Briefly, MDA-MB-435 cells grown on coverslips were treated with vehicle (0.2% DMSO) or 5 μM RQC for 48 h and stained with Annexin V-Cy3-18 in binding buffer (10 mM HEPES/NaOH, pH 7.5, 0.14 M NaCl, 2.5 mM $CaCl_2$) for 15 min at room temperature. Coverslips were washed in binding buffer and fixed with 3.7% paraformaldehyde prior to fluorescence microscopy. Images were digitally acquired from an Olympus inverted fluorescence microscope using Metamorph software (Molecular Devices, Sunnyvale, CA, USA) and quantified from ten random microscopic fields (20× mag.)/coverslip.

Cell migration

Equal numbers of viable quiescent GFP-tagged MDA-MB-231 or MDA-MB-435 cells (1×10^5) were placed in the top well of Transwell chambers where the bottom well contained vehicle (0.2% DMSO), 0.5 or 5 μM resveratrol, quercetin, or catechin or 0.5 or 5 μM RQC in serum-free and phenol red-free media. Following 8 h incubation, the cells on top of the membrane of the inner well were removed and the number of cells that migrated to the underside of the membrane through 8 μm diameter pores quantified following PI staining as described in [31].

Animals

Female athymic nu/nu mice, 5 6 week old (Charles River Laboratories, Inc., Wilmington, MA, USA) were maintained under pathogen-free conditions in Hepa-filtered cages under controlled light (12 h light and dark cycle), temperature (22 24°C), and humidity (25%). Throughout the experiment, the animals were provided with autoclaved AIN 76-A phytoestrogen-free diet (Tek Global, Harlan Teklad, Madison, WI, USA) and water ad libitum. This project was approved by the Universidad Central del Caribe Institutional Animal Care and Use Committee.

Tumor model

GFP-MDA-MB-435 cells ($\sim 1 \times 10^6$) in Matrigel (BD Biosciences, San Jose, CA, USA) were injected into the fourth right mammary fat pad of female nude mice under isoflurane inhalation to produce orthotopic primary tumors as described in [38]. After tumor establishment (1 week post-inoculation), the animals were randomly divided into experimental treatment groups. About 3 5 animals per group were eliminated due to failure of tumor take, small or too large tumor area in 1 week, or due to penetration of the peritoneum that resulted in immediate GFP fluorescence in the intestines. Mice with similar tumor area as quantified by integrated density of fluorescence images were selected for further study.

Diet administration

Nude mice ($n = 10$/experimental group) were orally gavaged either with vehicle (90% corn oil, 10% ethanol) or a combination of 5 mg/kg body weight (BW) resveratrol, 5 mg/kg BW quercetin, and 5 mg/kg BW catechin (RQC) in a 100 μl volume three times per week. The number of mice/group is in the range of previously published similar studies that demonstrated statistically significant differences in dietary treatments [39 41].

Whole body fluorescence image analysis

Mammary tumor growth was quantified as changes in intensity and integrated density of GFP-fluorescence as per our previously described methods [1, 42]. Anesthetized mice were imaged immediately following breast cancer cell inoculation and two times per week thereafter. A 300 Watt power source with two optical delivery systems fitted with excitation filters (470/40 nm) was used for whole body imaging of GFP fluorescence (LT99D2, Lightools Research, Encinitas, CA, USA). Images were captured with a Spot II charge-coupled device (CCD) camera (Diagnostic Instruments, Sterling Heights, MI, USA) mounted with a 530/25 nm emission filter (Chroma Technology, Rockingham, VT, USA).

Tumor fluorescence intensities were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The final images were acquired on day 77. Relative tumor area was calculated as the fluorescence intensity of each tumor on each day of imaging relative to the fluorescence intensity of the same tumor on day 1 of diet administration.

Analysis of metastases

Following sacrifice, lungs, kidneys, livers, femurs, and hearts were excised and immediately stored in liquid N_2. Stored organs were thawed and analyzed using an Olympus MV10 fluorescence macro zoom system microscope and images acquired with an Olympus DP71 digital camera. Each organ was imaged on both sides. The fluorescent lesions (green component of RGB images) were quantified for integrated intensity and area using Image J software.

Pixel values ranging from 0 to 255 were detected and a signal cut off of 58 (approximately one standard deviation above the mean of the maximum noise) was used to separate background signal from GFP signal. To eliminate potential false positives, a minimum fluorescent area threshold of 0.003 mm^2 was used (roughly four pixels). Areas identified as metastases were also validated by visual inspection and false positives eliminated from further analysis.

Real time reverse transcriptase (RT^2)-PCR analysis

At the end of the study, solid primary tumors at the mammary fat pad were immediately stored in "RNA later" (Ambion, Austin, TX, USA). Total RNA extraction and DNAase treatment was performed using the Qiagen RNeasy Kit (Qiagen, Valencia, CA, USA) following manufacturer's protocol. RNA concentration was detected using a NanoDrop (Thermo Scientific, Wilmington, DE, USA), while RNA integrity and quality analysis were evaluated using the Experion automated electrophoresis system (BioRad, Hercules, CA, USA). C-13 kit (SA Biosciences, Frederick, MD, USA) was used to synthesize cDNA from the extracted RNA (0.5 μg) and used to investigate gene expression profiles by the commercially-available phosphoinositide 3-kinase (PI3-K) Pathway Finder RT^2 Profiler™ PCR arrays (SA Biosciences, Frederick, MD, USA). This RT^2 Profiler™ PCR Array is designed to simultaneously profile the expression of 84 PI3-K pathway-specific genes, plus five housekeeping genes and seven RNA quality controls. The spreadsheets, gene tables, and template formulas included with the PCR array package were used to calculate relative changes in gene expression and perform statistical analyses. Reproducibility was maintained by using RNA from three tumors per treatment (three biological replicates).

Statistical analysis

Data are expressed as the mean ± SEM. Statistical analyses were done using Microsoft Excel or GraphPad Prism 5 software. Differences between means were determined using Student's *t*-Test and two-way ANOVA.

Results and discussion

Effect of grape polyphenols on metastatic breast cancer cells in vitro

Previously, we demonstrated that a combination of resveratrol, quercetin, and catechin at 0.5, 5, or 20 μM reduced cell number significantly from control and was more efficient than individual compounds in the MDA-MB-231 ERα (−) ERβ (+) human low metastatic breast cancer cell line [1]. However, due to the low metastatic nature of this cell line, we did not observe adequate metastases in a nude mouse model to enable a statistical analysis of the role of grape polyphenols on metastasis. Therefore, we tested the effect of dietary RQC on a highly metastatic ER (−) cancer cell line, MDA-MB-435. The origin of the MDA-MB-435 cell line has been called into question by several recent microarray studies that show expression of melanoma-associated genes [43]. However, MDA-MB-435 cells

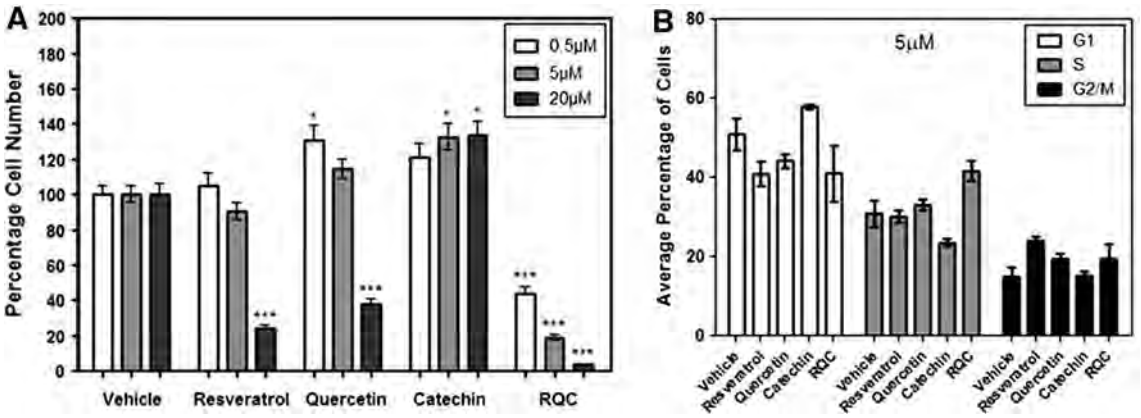

Fig. 1 Effect of grape polyphenols on MDA MB 435 cell proliferation and cell cycle progression. Cells in 5% serum and phenol red free media were treated with vehicle, 0.5, 5, or 20 μM resveratrol, quercetin, or catechin, or a combination of 0.5, 5, or 20 μM each (RQC) every 48 h for 96 h. Data was quantified from PI stained intact (non apoptotic) nuclei. **a** Cell proliferation. Percentage of viable cells ± SEM for 20 microscopic fields/triplicate treatments is presented. **b** Cell cycle progression. Cell cycle stage following 5 μM treatment with individual resveratrol, quercetin, or catechin or combined RQC. An *asterisk* indicates statistical significance of $P < 0.05$ and *three asterisks* indicate $P < 0.001$ when compared to vehicle

express breast differentiation-specific proteins and secrete milk lipids [44]. Since the patient had no evidence of melanoma but was diagnosed with only a breast carcinoma; and, since melanocytes do not produce milk, the simplest conclusion is that MDA-MB-435 is a very poorly differentiated breast carcinoma. This cell line has been extensively used to investigate metastasis from mammary fat pad inoculations, and remains as one of few models available for experimental metastasis of breast cancer in nude mice [33, 45].

As shown in Fig. 1a, 0.5 or 5 μM treatment with resveratrol, quercetin, or catechin alone did not decrease MDA-MB-435 cell proliferation. Resveratrol or quercetin at high concentrations (20 μM) significantly inhibited cell proliferation by 80 and 60% while catechin alone increases cell proliferation significantly. Therefore, the effects on cell proliferation at 20 μM RQC appear to be an additive effect of resveratrol and quercetin. The combined RQC treatment significantly inhibited MDA-MB-435 cell proliferation by ~50, 80, and 90% at 0.5, 5, or 20 μM of each polyphenol (Fig. 1a). These compounds were more effective in the MDA-MB-231 cell line where both resveratrol and quercetin inhibited cell proliferation at 5 and 20 μM by ~60 and ~95%; while combined resveratrol, quercetin, and catechin (RQC) treatment at 0.5, 5, and 20 μM each inhibited cell proliferation by ~60, 85, and 98%, respectively, compared to vehicle controls [1]. Since combined RQC treatment induced a significant reduction on MDA-MB-435 cell proliferation, we then tested the cell cycle stage of MDA-MB-435 cells following 5 μM RQC treatment and found that these compounds arrested MDA-MB-435 cells at S phase as was shown before with MDA-MB-231 cells (Fig. 1b), [1]. However, unlike with the MDA-MB-231 cells, the S phase arrest of the MDA-MB-435 cells in response to RQC demonstrated a P value of 0.06.

The method we used to recover cells for analysis of cell cycle stage (i.e., trypsinization followed by centrifugation, fixing, staining, and washing) does not account for potentially apoptotic, non-adherent, or weakly adherent cells that may become removed during the repeated washings. Moreover, the observed increase in cells at S-phase does not correlate with the 80% decrease in cell number observed with 5 μM RQC (Fig. 1a, b). Therefore, we investigated the effect of RQC treatment on apoptosis of MDA-MB-435 cells by caspase 3 activity. This downstream effector caspase was selected to assess the effect of RQC on both receptor-regulated and mitochondrial apoptotic pathways. As shown in Fig. 2a, 5 μM RQC treatment increased caspase 3 activity by twofold at a $P < 0.06$ when compared to vehicle, while RQC at 0.5 μM did not affect caspase 3 activity in a significant manner. Similarly, Annexin V staining of MDA-MB-435 cells to detect phosphatidyl serines on the outer

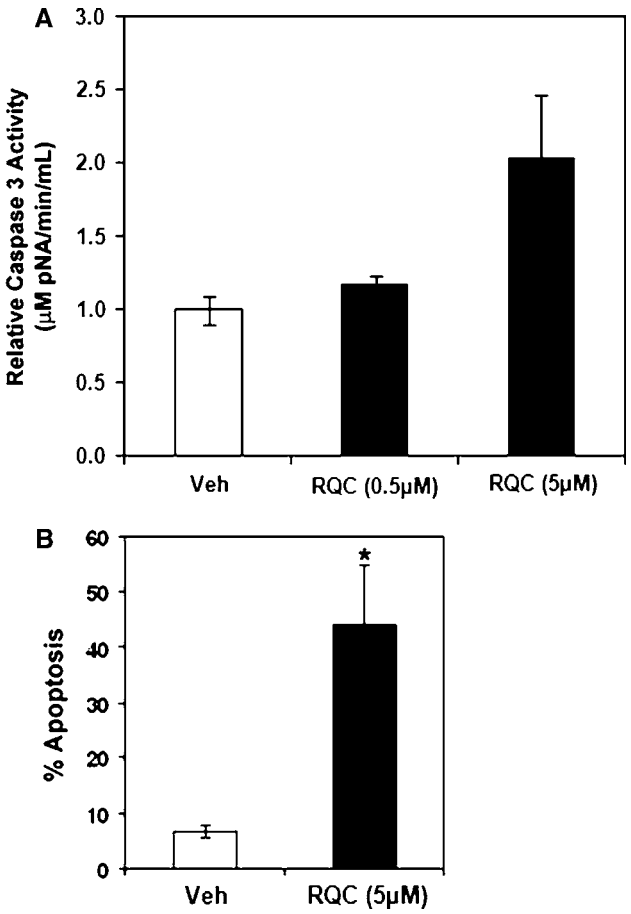

Fig. 2 Effect of grape polyphenols on apoptosis of MDA MB 435 cells. Apoptosis of MDA MB 435 cells was detected by caspase 3 activity assays (**a**) or fluorescence microscopy for Annexin V staining (**b**) following 48 h incubation with vehicle or RQC. **a** Average caspase 3 activity in μmol of pNA min^{-1} ml^{-1} relative to vehicle (n 4 ± SEM) as quantified from absorbance at 405 nm of the pNA released by caspase 3 activity. **b** Percentage of cells undergoing apoptosis was calculated by Image J analysis of brightfield (total number of cells) and red fluorescence (apoptotic cells stained with Annexin V Cy3) from ten random microscopic fields/coverslip. Averages ± SEM are shown for two separate experiments with duplicates for each experiment (n 4). An *asterisk* indicates statistical significance ($P < 0.05$) when compared to vehicle

leaflet of the cell membrane indicated that at 48 h following 5 μM RQC, 44% of cells were significantly apoptotic ($P < 0.01$) compared to only 6.8% of vehicle-treated cells (Fig. 2b). Resveratrol and quercetin at high concentrations have been implicated in apoptosis of cancer cell lines by inducing caspase activity and inhibition of cell survival via PI3K/Akt pathways [23, 46, 47]. In MDA-MB-231 cells, by western blotting with total Akt and phospho-$Akt^{ser\text{-}473}$ antibodies, 5 μM RQC (15 min) was found to decrease Akt activity by ~50% compared to vehicle (data not shown). Therefore, the observed decrease in breast cancer cell numbers in response to RQC treatment is thought to be due

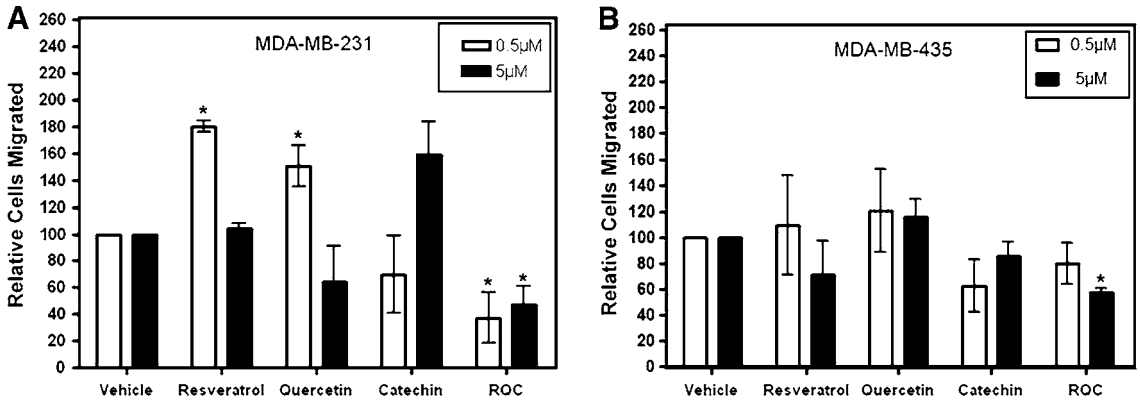

Fig. 3 Effect of grape polyphenols on breast cancer cell migration. Quiescent MDA MB 231 (**a**) or MDA MB 435 (**b**) cells were placed on the top well of Transwell chambers in serum free, phenol red free media and the number of cells that migrated through the membrane of the top well in response to various treatments was quantified relative to control. Data are quantified from analysis of 25 microscopic fields/treatment ($n \quad 3 \pm$ SEM). The bottom well contained the following for 8 h: vehicle, 0.5 or 5 μM resveratrol, quercetin, catechin or a combination of 0.5 or 5 μM each (RQC). An *asterisk* indicates statistical significance ($P < 0.05$) when compared to vehicle

to a block in cell cycle progression, increased apoptosis, and reduced cell survival signaling.

Since directed cell migration has been implicated with metastatic efficiency, we tested the effect of individual and combined grape polyphenols on cell migration. Migration assays were performed using Transwell chambers where individual resveratrol, quercetin, or catechin or combined RQC treatment was added to the bottom well while the inner well contained serum-starved MDA-MB-231 or MDA-MB-435 cells. In MDA-MB-231 cells, resveratrol and quercetin at 0.5 μM increased cell migration in a statistically significant manner (Fig. 3a). The effect of resveratrol is similar to our previous results that reported low concentrations of resveratrol to act comparable to estrogen and increase cell migration [31]. None of the other grape polyphenols significantly changed breast cancer cell migration. At 0.5 and 5 μM, combined RQC treatment significantly reduced MDA-MB-231 cell migration by ~60% when compared to vehicle controls; whereas, 0.5 μM combined RQC treatment reduced MDA-MB-435 cell migration by ~20%, and 5 μM RQC significantly reduced cell migration by 40% (Fig. 3).

The lower response of the inhibitory effect of RQC treatment in the ER (−) MDA-MB-435 cells compared to the ERβ (+) MDA-MB-231 cells may be due to grape polyphenols acting as antiestrogenic compounds in the MDA-MB-231 cells. Also, since MDA-MB-435 cells are $Her2^{++}$ it is possible that combined grape polyphenols are not as efficient at inhibiting the increased Her-2 signaling in this highly aggressive cancer cell line. However, mechanistic studies need to be conducted to further address the differences in response to grape polyphenols between these two cancer cell lines.

Effect of grape polyphenols on mammary tumor growth in vivo

To test the effect of resveratrol, quercetin, and catechin on metastatic breast cancer progression in vivo, we established mammary fat pad tumors from GFP-tagged highly metastatic MDA-MB-435 cancer cells as previously described [42]. As quantified from the integrated density of fluorescent images, mice ($n = 10$ per group) with similar initial tumor volumes (vehicle group = 9,036.6 ± 654 and RQC group = 9,825 ± 501) were selected for further study.

One week following tumor establishment, mice were gavaged with vehicle (90% oil, 10% ethanol) or 5 mg/kg BW RQC three times a week. This dietary concentration was selected based on our previous study where administration of 0.5, 5, or 20 mg/kg BW RQC demonstrated that the inhibitory effect on mammary tumor growth plateaued at 5 mg/kg BW [1]. Tumor progression was quantified by fluorescence image analysis twice a week. The relative tumor area was calculated as the fluorescence intensity of each tumor on day of imaging relative to the fluorescence intensity of the same tumor on day 1 of diet administration as described in [1]. As shown in Fig. 4a, tumor growth remained linear and similar for both vehicle and RQC treated mice for 60 days. After 60 days, the RQC-treated mice demonstrated reduced tumor growth compared to vehicle. At the end of the study on day 77, the mice following RQC diet demonstrated smaller tumors that were reduced by ~37% in a statistically significant manner (Fig. 4b). Previously, we reported a 69% decrease in MDA-MB-231 mammary tumor growth with 5 mg/kg BW RQC treatment [1]. The present data demonstrates that, as with the in vitro effects, dietary RQC treatment of nude

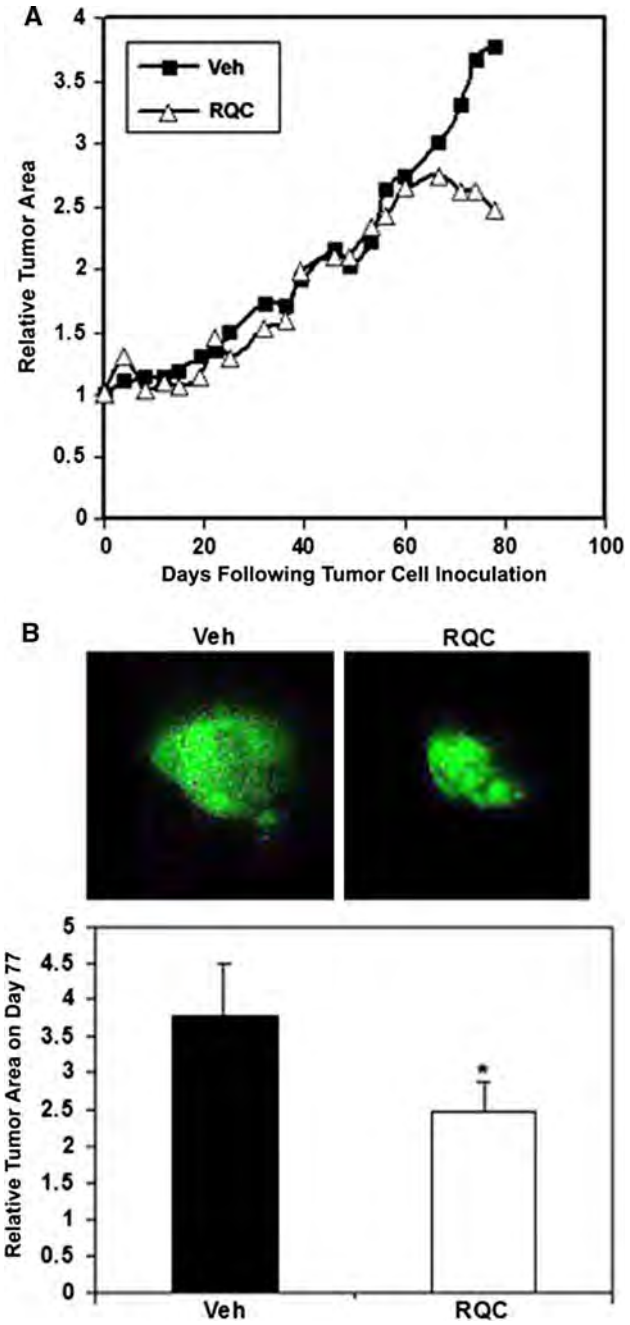

Fig. 4 Effect of grape polyphenols on the growth of MDA MB 435 mammary fat pad tumors. MDA MB 435 cells (10^6) in Matrigel were inoculated at the mammary fat pad of nude mice. One week following injection, mice were fed vehicle or a combination of 5 mg/kg BW Res, 5 mg/kg BW Quer, and 5 mg/kg BW Cat (RQC) three times a week by oral gavage. Whole body fluorescence images were acquired two times a week. **a** Average relative tumor area as a function of days following injection. Relative tumor area was calculated as the area of fluorescence, measured by fluorescence intensity on each day of imaging as a function of the fluorescence intensity of the same tumor on day 1. **b** GFP MDA MB 435 tumors following vehicle or RQC diets at day 77. Representative digital images and mammary tumor area as quantified from digital images acquired on day 77, made relative to same tumor image on day 1. *Asterisk* denotes statistical significance at $P < 0.05$

mice with MDA-MB-435 mammary tumors results in a significant inhibition of primary tumor growth but this effect is less compared to the effect of RQC treatment on MDA-MB-231 mouse mammary xenografts.

At the end of the study (77 days), there were no statistically significant differences in body weights from mice treated with vehicle (24.35 g ± 1.6) or RQC (24.04 g ± 1.7). This is similar to our previous report where 118 days of dietary RQC treatment at concentrations as high as 25 mg/kg BW did not significantly change mouse weights from vehicle controls [1]. Therefore, the decrease in tumor area at the end of the study was not due to toxic effects of dietary RQC. This data indicates that combined grape polyphenols can be safe and effective therapeutics and preventives of primary tumor growth of ER (−) breast cancer.

To initiate a molecular analysis of the effect of grape polyphenols on breast cancer, we analyzed changes in expression of PI3-K pathway genes because this pathway is a central regulator of cancer cell survival and invasion [48]. Interestingly, real-time PCR arrays for PI3-K pathway genes from tumor extracts revealed that expression of FOXO1 transcription factor was upregulated significantly by 1.87-fold in mouse mammary tumors following RQC treatment (Table 1). FOXO factors have been shown to function as tumor suppressors in a variety of cancers. They are actively involved in promoting apoptosis in a mitochondria-independent and -dependent manner by inducing the expression of death receptor ligands and pro-apoptotic Bcl-2 family members [35]. Forkhead proteins have been shown to be important for the anticancer activities of resveratrol [49]. This is the first time that elevation of death promoting genes have been implicated in vivo for a combination of dietary grape polyphenols in reducing mammary tumor growth. Since FOXO1 can be inactivated via Akt-mediated phosphorylation, elevation of FOXO1 transcripts may not necessarily result in increased protein activity. Interestingly, AKT1 expression was also decreased by threefold in the PCR array but this was not statistically significant. Moreover, RQC treatment of breast

Table 1 Effect of RQC treatment on expression of PI3 kinase pathway genes

Gene	Fold difference RQC/vehicle	*P* value
FOXO1, forkhead box 01A	1.87	0.007
NFKBIA, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	1.70	0.041
TLR4, toll like receptor 4	1.91	0.003

Only genes that demonstrated >1.5 fold difference and *P* value of <0.05 from RT^2 PCR arrays are shown

cancer cells significantly reduces active phospho Akt1 (*p*-Akt $^{ser\ 473}$) levels in 15 min without affecting total Akt1 levels (data not shown). The relative contribution of decreased phospho Akt1 levels and increased FOXO1 levels and caspase 3 activity to RQC-mediated effects on cell survival and apoptosis is currently under investigation.

NfκB transcription factor that regulates tumorigenic and immunomodulatory signaling is a potential target for the chemopreventive activity of grape polyphenols, resveratrol, and quercetin [50 53]. NFKBIA, which codes for IκB, the subunit that sequesters NfκB in an inactive state, was also upregulated significantly by 1.7-fold in mammary tumors following RQC treatment (Table 1). This data indicates that inhibition of NfκB signaling may contribute to the observed reduced mammary tumor growth and metastasis by grape polyphenols. However, IκB proteins can become inactivated via phosphorylation-induced, proteasome-mediated degradation by IκB kinase (IKK) activity. Therefore, increased NFKBIA gene expression by RQC treatment may not reflect increased stable protein levels. Future experiments will determine the stability and phosphorylation status of IκB in response to RQC. In addition, we also found a significant increase in Toll-like receptor 4 (TLR4) expression that have been implicated in cancer progression (Table 1). However, TLRs may also stimulate apoptosis under certain conditions [54] and can be negatively regulated by PI3-K signaling [55]. Therefore, the significance of this result warrants further investigation.

Previous in vivo studies have also supported a role for grape polyphenols in cancer prevention. Grape juice, grape seed extract, and red wine have been shown to significantly reduce cancer in rodent models [56 58]. Grape skin extract, which is concentrated in red wine, was recently shown to contain more growth inhibitory effects than grape pulp, juice, or seeds on mouse mammary tumor growth [59]. Since the effect of grape polyphenols on cancer metastasis remains to be evaluated, we next analyzed the effect of dietary grape polyphenols on breast cancer metastasis.

Effect of grape polyphenols on metastasis

Our macro imaging system easily detected surface primary tumors, local invasion into the circulatory system, lymphatics, and metastatic tumors in the GI tract through the skin of nude mice. However, the resolution of this imaging system allows detection of only $\sim 10^4$ GFP-tagged cells and is thus limited in sensitivity for detection of micrometastases. Therefore, fluorescent metastatic lesions were quantified by microscopy following surgical removal of organs. Only eight mice/treatment were used for analysis of metastasis due to early death of two from the vehicle group and one from the RQC group. This number is similar to a previous study that reported the effect of dietary genistein on metastasis from MDA-MB-435 mammary tumors [39]. All of the mice following vehicle or RQC treatment presented with lung metastases. Therefore, lungs were further analyzed by Image J for quantification of the area of fluorescence. The number of metastatic lesions/lung was reduced in RQC-treated mice in a statistically significant manner when compared to vehicle treatment. However, the area of fluorescence calculated from these lesions was not statistically different for mice treated with vehicle or RQC (Fig. 5a, b, c; Table 2). Therefore, we conclude that RQC treatment does not block metastases to the lung from this cancer cell variant.

The MDA-MB-435 cell line used in this study was selected as a bone metastatic variant by intracardiac injection in nude mice [33]. Since breast cancers preferentially metastasize to the bone [60], this trend was simulated by inoculating the MDA-MB-435 bone metastatic variant into the mammary fat pad. The ability of the GFP-MDA-MB-435 mammary tumors to invade bone was investigated by fluorescent image analysis of excised, cleaned femurs from mice

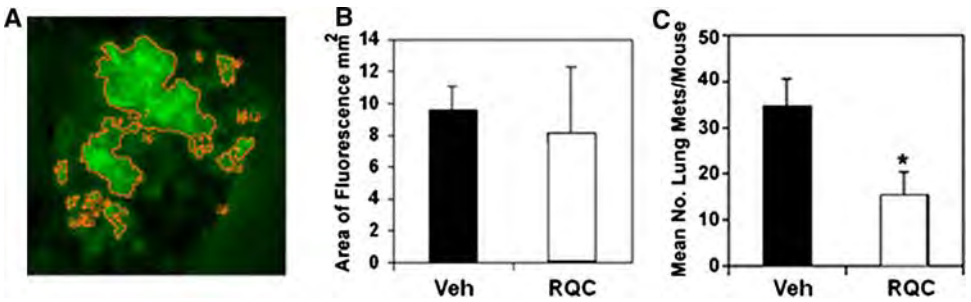

Fig. 5 Effect of grape polyphenols on lung metastasis. Following necropsy, lungs were excised from mice with GFP MDA MB 435 mammary tumors that received either vehicle or RQC diets and analyzed for metastases by fluorescence microscopy followed by quantitative image analysis. **a** Green fluorescence image of a representative lung demonstrating analysis of traced fluorescence area. **b** Lung metastatic efficiency expressed as average area of fluorescence from lungs of vehicle or RQC treated mice ±SEM (n 8). **c** Average number of fluorescent metastatic foci/lung for vehicle or RQC treated mice. *Asterisk* denotes statistical significance at $P < 0.05$

Table 2 Distant metastases in mice following vehicle or combined resveratrol, quercetin, and catechin (RQC) treatment

	Number of mice with metastases (*N* 8)		Number of metastatic lesions/organ with metastases	
	Veh	RQC	Veh	RQC
Bone	5	2	11.4	3
Heart	4	1	1.5	1
Kidney	3	3	15.7	1.33
Liver	8	2	22	11
Lung	8	8	34.7	15.7
Lymph node	3	3	2	1.7

on vehicle control or RQC diets as described in [33]. In vehicle controls, 5/8 mice presented with bone metastases while only 2/8 mice following RQC treatment demonstrated fluorescent metastatic foci in femurs. Of the mice with bone metastases, the number of metastatic lesions were higher for vehicle treated mice compared to RQC treatments in a statistically significant manner (Fig. 6a, b; Table 2). Similarly, the number of mice with liver metastases and the number of metastatic lesions/liver were also significantly lower in RQC treated mice compared to vehicle controls (Fig. 6c, d; Table 2). The mean fluorescent area or integrated density for a single metastatic lesion were similar for bone or liver metastases from vehicle or RQC treated mice (data not shown); however, very few the RQC-treated mice presented bone or liver metastases and exhibited lower numbers of metastatic foci/organ.

Table 2 also shows that less RQC treated mice presented with heart metastases when compared to vehicle treated mice. However, the number of metastatic foci/heart in RQC treated mice was only slightly less when compared to vehicle. When kidneys were examined for metastases, the number of mice with kidney metastases did not change but there were more metastatic lesions/kidney in the vehicle treated mice. Only three mice for vehicle or RQC treatments demonstrated lymph node metastases. Since the numbers of mice with heart, kidney, or lymph node metastases were low even for vehicle treatments, it is not possible to analyze these results in a statistically significant manner or derive definitive conclusions on the effect of RQC treatment compared to controls.

To our knowledge, this is the first report of an inhibitory effect of grape polyphenols on breast cancer metastasis. The differential effects of RQC on site-specific metastases

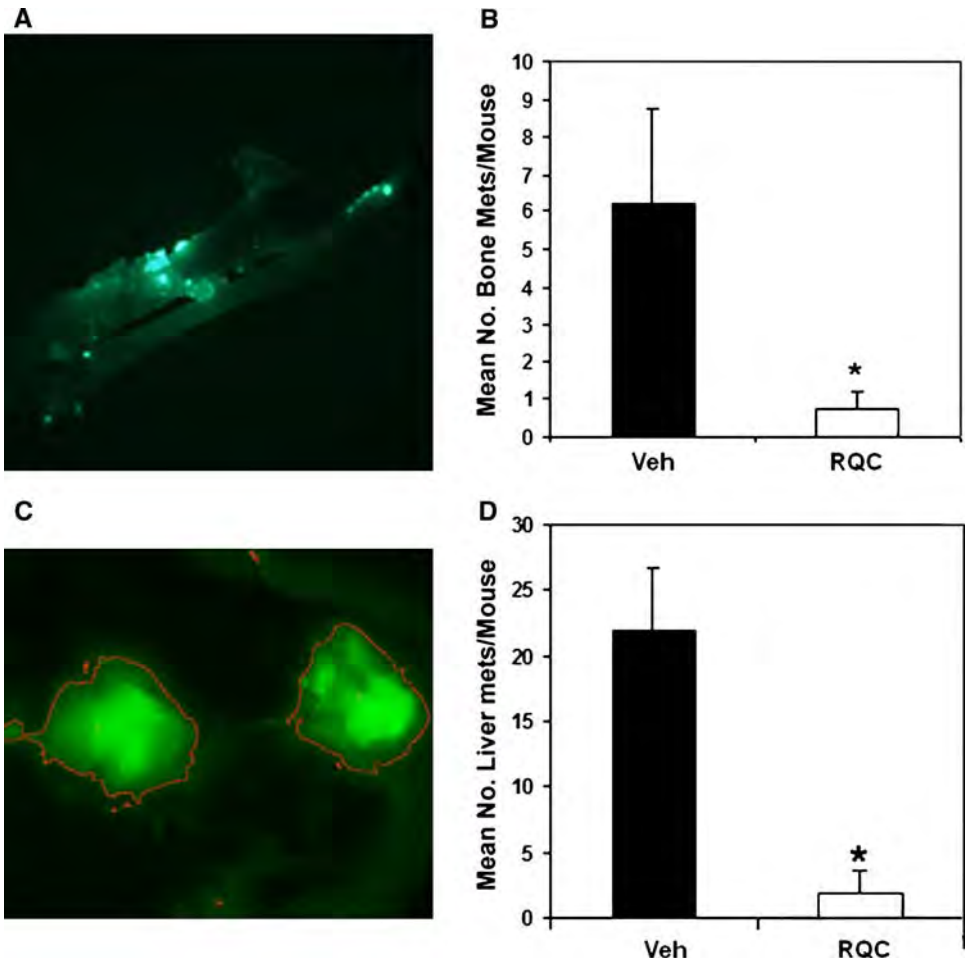

Fig. 6 Effect of grape polyphenols on bone and liver metastasis. Following necropsy, femurs and livers were excised from mice with GFP MDA MB 435 mammary tumors that received either vehicle or RQC diets and analyzed for metastases by fluorescence microscopy followed by quantitative image analysis. **a** Green fluorescence image of a representative femur from vehicle treated mouse. **b** Average number of fluorescent metastatic foci/femur for vehicle or RQC treated mice. **c** Green fluorescence image of a representative liver from vehicle treated mouse. **d** Average number of fluorescent metastatic foci/liver for vehicle or RQC treated mice. *Asterisk* denotes statistical significance at $P < 0.05$

indicate that RQC treatment did not inhibit metastatic cancer cells from being released to the lymphatics or the vascular system from the primary tumors at the mammary fat pad. RQC treatment also did not block the entry of cells into the lungs, where all of the mice in the study presented with lung metastases. Interestingly, subsequent metastases to the bone and liver were reduced by RQC treatment, indicating that these compounds may affect establishment of further metastases either by regulation of exit from the lung vasculature or at the entry points of localized signaling at liver and bone microenvironments. Our intriguing data that demonstrates upregulation of NFKBIA levels in mammary tumors following RQC treatment implicates inhibition of NfκB signaling by dietary grape polyphenols as a potential pathway that regulates breast cancer progression. Interestingly, NfκB signaling has been associated with bone and liver metastasis [61 63]. The mechanistic basis for these interesting possibilities and the ability of grape polyphenols to specifically inhibit components of the bone and (or) liver molecular signature are currently under investigation.

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Title: **Novel Inhibitors of Rac1 as Anti Breast Cancer Metastasis Compounds**

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Novel Inhibitors of Rac1 as Anti Breast Cancer Metastasis Compounds

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Abstract

Background: Rho family GTPases are molecular switches that control signaling pathways regulating a myriad of cellular functions. Rac1, a Rho family member, plays a critical role in several aspects of tumorigenesis, cancer progression, invasion, and metastasis. Rac proteins are not mutated in most invasive human cancers but are found to be overactive or over-expressed. Since Rho GTPases are activated by guanine nucleotide exchange factors (GEFs), inhibition of the interaction of Rac with its GEFs is a targeted strategy for blocking Rac activation.

Methods: The IC_{50} of NSC23766, an inhibitor of the interaction of Rac1 with a subset of GEFs, is too high for therapeutic use and more efficacious inhibitors are desired. Therefore, we initiated the synthesis of new derivatives of NSC23766 with modifications of the substituents connected to the central pyrimidine ring, and tested their Rac1 inhibitory activity.

Results: Several of the NSC23766 derivatives were shown to inhibit Rac1 activity of cancer cells with higher efficiency than NSC23766. The new compounds are not toxic to normal mammary epithelial cells and are more efficient than NSC23766 in inhibiting cell migration and reducing cell spreading and extension of lamellipodia, cell functions regulated by Rac that contribute to cancer invasion.

Conclusions: Based on the results, we concluded that the novel compounds show promise of further development as small molecule inhibitors of invasive breast cancer progression.

*Corresponding authors

Introduction

Breast cancer is the leading cancer type estimated for 2009 for US women and ranks first for death from all cancers at ages 20-59 (1). Early detection and aggressive breast cancer therapy in the US has resulted in a recent decline in mortality rates from breast cancer. However, in Puerto Rico, breast cancer mortality rates are rising and breast cancer has become the most frequently diagnosed form of cancer in women. Moreover, the recently released 2006 cancer statistics from the Centers for Disease Control and Prevention report that breast cancer is the number one killer of Hispanic women from all cancer related deaths. Breast cancer is most deadly when metastasis to other tissues occurs and novel therapies to inhibit spreading of the tumor are highly desired (2). We investigated Rac1 as a key protein involved in signaling pathways for metastasis, and synthesized novel small molecule compounds that can interfere with this process.

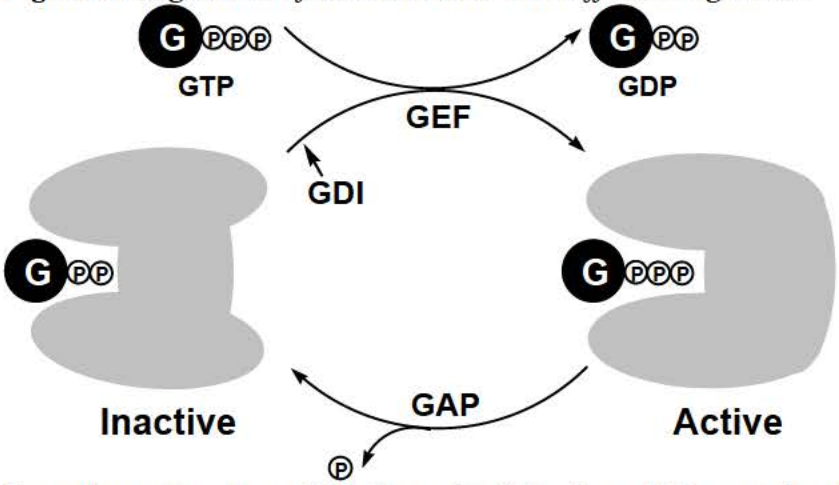
The involvement of Rac1 in metastatic processes

In metastasis, malignant cells migrate away from the original tumor site to other parts of the body. An important stage in the movement of these cells is the extension of actin-based protrusions by means of membrane ruffling and the formation of lamellipodia. Lamellipodia are sheet-like extensions of cross-linked networks of polymerized actin, and enable forward migration of the cells during invasion (3) and thus, regulate metastasis (4). A critical step for initiation of the cytoskeletal remodeling involved with lamellipodia formation is the activation of the small (21kD) protein Rac1 (5). Therefore, inhibition of Rac1 activity could impede cell migration and provide a potential approach to prevent and halt metastasis (6).

Rho GTPases

Rac1 belongs to the Rho family of GTPases, of which thus far 22 members have been identified (7). Rho GTPases are important intracellular signaling proteins that control diverse cellular functions related to cancer development, including actin cytoskeleton organization, invasion and metastasis, transcription regulation, cell cycle progression, apoptosis, vesicle trafficking, and cell-to-cell and cell-to-extracellular matrix adhesions. Rho GTPases can be present in either a GDP-bound inactive conformation as well as in a GTP-bound active conformation, and is regulated mainly via GEFs (Guanine nucleotide exchange factors), GDIs (Guanine nucleotide dissociation inhibitors) and GAPs (GTPase activating proteins) (figure 1) (8). When a GEF binds to a Rho GTPase in its GDP-bound inactive state, a molecule of GDP is exchanged for GTP, upon which a conformational change occurs that brings the Rho GTPase into an active state. For some RhoGTPases GDIs have been identified that inhibit this exchange of GDP for GTP. In its GTP-bound active conformation, the Rho GTPase can bind to its down-stream effectors, which mainly via activation of kinases leads to a biological effect. On the other hand, when a GAP interacts with active Rho GTPase, GTPase activity is stimulated and the bound GTP is hydrolyzed to GDP, thus reestablishing the Rho GTPase in its inactive form. Each member of the Rho GTPase class is regulated by one or more GEFs or GAPs.

Figure 1: *Regulation of Rho GTPases with different regulators*



For explanation: see text. GDP: Guanosine diphosphate; GTP: guanosine triphosphate; GEF: Guanine nucleotide exchange factor; GDI: Guanine nucleotide dissociation inhibitor; GAP: GTPase activating protein; P: phosphate.

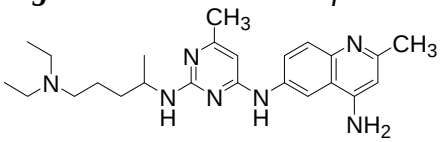
Rac1 as a target for drug discovery

The three main Rho GTPases are Rac1, Cdc42 and RhoA, and each has been shown to be specifically involved in lamellipodia formation, filopodia formation and stress fiber formation respectively (3,7). For both Rac1 and Cdc42, evidence has been obtained for their role in tumor growth, invasion and cell migration in breast tumors. Alongside Trio and Vav (9), an important GEF of Rac1 is "T-cell lymphoma invasion and metastasis 1" (Tiam1), which is considered to be an invasion-inducing gene (10). Increased expression of Rac1 has been reported in breast, colon and lung tumors (11), while over-expression of Tiam1 has been reported in highly invasive breast tumors and colon carcinomas (12). Increased Trio, Vav1, and Tiam 1 expression has been associated with high grade breast tumors (13). In addition, we and others have implicated hyperactive Rac1 with increased proliferation and invasion of breast cancer (14). Therefore, prevention of the binding of Tiam1 to Rac1 appears to be an attractive approach to inhibit the formation of lamellipodia and cell migration implicated with metastatic tumor cells.

Identification of a lead structure

The crystal structure of Rac1 with its GEF Tiam1 has revealed specific binding site residues required for binding of Rac1 with Tiam1 (15). Recently, via a virtual screening procedure utilizing molecular docking of the NCI library in this binding site, the group of Zhang identified NSC23766 (compound **1,** figure 2) as a selective inhibitor of Rac1 (16). NSC23766 has been shown to inhibit Rac activity and cell invasion from a number of systems (17). However, in breast cancer, NSC23766 only inhibited the invasion of low metastatic human breast cancer cell lines and not high metastatic cells. As we were interested in compounds that interfere with metastatic processes, we investigated the Rac1 inhibitory activity of NSC23766 in the highly metastatic cell line MDA-MB-435. In this cell line, even at a concentration up to 200µM of compound **1**, only 40% inhibition of Rac1 activity was observed. Therefore, we set out to utilize **1** as a lead structure for the synthesis of new derivatives with the potential for improved Rac1 inhibitory activity.

Figure 2: *Lead structure for inhibitor design*



1 (NSC23766)

Materials and Methods

Syntheses

Materials*:* All building blocks were purchased from Sigma-Aldrich Chemical company. ***Procedures:*** All novel NSC23766 derivatives were synthesized according to the reaction scheme provided in scheme 1. As a representative example, the detailed two-step procedure for the synthesis of compound **7** is provided***. Step 1:*** *Synthesis of (2-Chloro-6-methyl-pyrimidin-4-yl)-(2,3-dihydro-benzothiazol-6-yl)*-amine (compound **8**) To a solution of 1.63 g (10.0 mmol) 2,4-dichloro-6-methylpyrimidine in 10 mL *iso*-propanol, 1.97 mL (11.0 mmol) diisopropyl ethylamine (DIPEA) and 1.65 g (11.0 mmol) 6-aminobenzothiazole were added. The solution was refluxed for 18 hours, after which the starting materials were shown to be consumed as detected by TLC (thin layer chromatography) or by GC/MS (Gas Chromatography with Mass Spectrometry detector). After the reaction mixture was cooled to room temperature, 20 mL ethyl acetate and 10 mL distilled water were added, and after vigorous mixing, the layers were separated with the aid of a separation funnel. The organic layer was extracted with 10 mL brine, separated and dried on sodium sulfate, filtered and concentrated on a rotary evaporator to obtain 2.49 g (9.0 mmol = 90%) of a crude yellowish solid. According to GC/MS a 3.0 : 1.0 mixture of respectively the 4-substituted and 2-substituted regioisomers was obtained. After silica gel chromatography using 3 : 1 hexanes/ethyl acetate as the eluent, the 4-substituted compound **8** was obtained as a pure regioisomeric compound in a yield of 1.11 g (4.0 mmol = 44% yield from crude material). The product was identified with NMR and GC/MS: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.40 (s, 3H), 6.61 (s, 1H), 7.65 (d, *J* = 8.8 Hz, 1H) 8.11 (d, *J = 10.5*, 1H), 8.49 (s, 1H), 9.30 (s, 1H), 10.25 (s, 1H); ^{13}C (DMSO-d_6, 100 MHz) δ 23.2,

103.7, 112.9, 120.1, 12.9, 123.1, 149.2, 154.7, 158.9, 161.8, 167.3; LRGC-MS *m/z* (rel%): $[M]^+$ 276 (100), $[M-Cl]^+$ 241 (40), $[M-C_5H_5N_3Cl]^+$ 134 (26). ***Step 2:*** *Synthesis of N4-Benzothiazol-6-yl-N2-(4-diethylamino-1-methyl-butyl)-6-methyl-pyrimidine-2,4-diamine* (compound **7**). In the second step, in a 10 mL glass microwave tube, 0.28 g (1.0 mmol) of compound **8** obtained in step 1 was dissolved in 1.0 mL *sec*-butanol, and 522 µL (3.0 mmol) DIPEA and 400 µL (2.0 mmol) 2-amino-5-diethylaminopentane were added. The tube was capped, placed in a CEM microwave synthesizer and heated to 120 ºC for 30 minutes. After the reaction mixture was cooled to room temperature, 10 mL ethyl acetate and 5 mL distilled water were added, and after vigorous mixing, the layers were separated with the aid of a separation funnel. The aqueous layer was extracted three times with 10 mL ethyl acetate, and after combining the organic phases they were extracted with 5 mL brine. The organic phase was separated and dried on sodium sulfate, filtered and concentrated on a rotary evaporator to obtain an off-white solid that was purified via silica gel chromatography to obtain 0.32 g (0.81 mmol = 81%) of product **7**. The product was identified to be essentially pure by NMR and GC/MS analysis: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.10 (t, *J = 7.1* Hz, 9H), 1.35 (d, *J = 6.4* Hz, 3H), 1.5-1.8 (m, 2H) 2.80 (m , 1H), 3.40 (s, 1H), 4.15 (m, 2H), 6.0 (s, 1H), 7.65 (d, J = 8.6 Hz, 1H), 7.96 (d, J = 8.8 Hz, 1H), 8.7 (s, 1H), 9.11 (s, 1H); ^{13}C (DMSO-d_6, 100 MHz) δ 9.9, 21.75, 22.8, 23.6, 35.5, 47.5, 48.2, 53.3, 96.5, 113.2, 121.1, 123.7, 135.9, 140.2, 149.5, 155.0, 163.2, 166.8; LRGC-MS *m/z* (rel%) $[M]^+$ 398 (4), $[M-CH_2CH_3]^+$ 369 (6), $[M-C_5H_{12}N]^+$ 86 (100).

Cell culture

MDA-MB-435 human metastatic breast cancer cells and MCF-10A mammary epithelial cells were purchased from ATCC and cultured in 10% DMEM at 5% CO_2 and 37ºC incubator as described in (14c,d). The origin of MDA-MB-435 cell line has been questioned by comparative genomic hybridization studies that report MDA-MB-435 and M14 melanoma to be identical cell lines. However, as reviewed in (18), both cell lines may be of MDA-MB-435 breast cancer origin rather than of melanoma origin due to the following rationale. The MDA-MB-435 cell line was isolated from a pleural effusion of a female patient with breast cancer and still has two X chromosomes; expresses milk proteins and lipids; and when transfected with the nm23 metastasis suppressor gene, MDA-MB-435 cells show the morphologic features of normal breast epithelial cells, including acinus formation in three-dimensional culture. Therefore, the MDA-MB-435 cell line was used as a model for a highly invasive and metastatic breast cancer with high Rac activity.

Rac activity assay

MDA-MB-435 cells were treated with vehicle (0.1% DMSO), or 50 µM Rac Inhibitor for 24 hrs, and total protein extracts were obtained using the lysis protocol and buffers in the G-LISA Rac1 Activation Assay (Cytoskeleton, Inc., Denver, CO). As per manufacturer's instructions, Rac activity was measured in a 96 well plate coated with the Rac.GTP binding domain of p21-activated kinase (PAK) using the GLISA colorimetric assay.

Cell viability assay

Plates were seeded on a 96 well plate at a concentration of 2,000 cells/well in 100 µl of Phenol Red (-) DMEM with 5% fetal bovine serum (FBS). Cells were incubated in vehicle (0.1% DMSO) or Rac inhibitors at 50 µM for 24 hrs. Cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cell survival and proliferation kit (Millipore, Inc., Billerica, MA). Absorbance at 600 nm of each experimental sample (3 biological repeats with 7 technical repeats), was measured from a 96 well plate on a Microplate Reader (Bio-Rad). The mean values ± SEM (N=3) are presented relative to vehicle (100%).

Cell migration assays

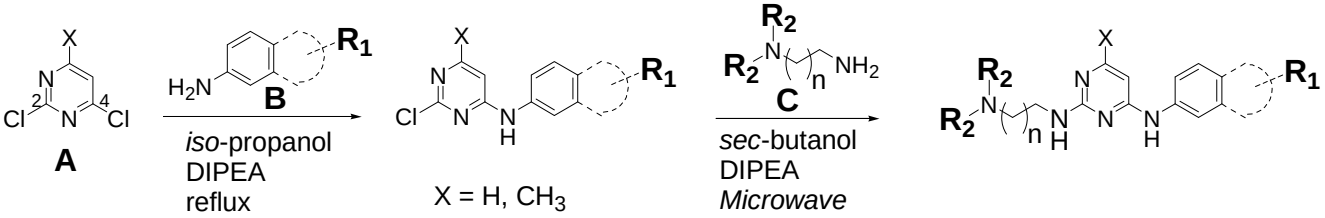
Quiescent MDA-MB-435 breast cancer cells were treated with vehicle (0.1% DMSO), or 50 µM Rac Inhibitor for 24 hrs. Cells were trypsinized and placed on the top well of Transwell chambers (Corning Life Sciences, Lowell, MA) in serum-free media with vehicle or 50 µM inhibitors. The bottom well contained 10% serum. The number of cells that migrated through 8µ pores in the membrane of the top well in response to various treatments was quantified relative to vehicle (100%) after 4 hrs. Data (±SEM) were quantified from analysis of 20 microscopic fields/treatment from 3 biological replicates.

Fluorescence microscopy
Quiescent GFP-tagged MDA-MB-435 cells were treated with vehicle (0.1% DMSO) or 50 µM Rac Inhibitors for 24 hrs. Cells were fixed and stained for polymerized actin with Rhodamine Phalloidin as described in (14c). Representative microscopic fields are shown at 60X magnification.

Results and Discussion

Synthetic design
Compound **1** and analogous derivatives can be synthesized via successive connection of three chemical building blocks. The central building block is a pyrimidine core (**A**), which has been suggested to bind to a critical tryptophane (Trp56) residue of Rac1 (19). The second building block has a hetero-bicyclic arylamino group (**B**) connected to the 4-position of the pyrimidine ring, and the third building block is a primary or secondary aliphatic amine with a tail-end amino-group (**C**) connected to the 2-position of the pyrimidine ring. We developed a reaction scheme for the straightforward synthesis of a number of derivatives of compound **1** that allowed for variation in the building blocks (Scheme 1). In the first step, 2,4-dichloropymidine **A** is reacted with the arylamine **B** to give a 3:1 to 5:1 mixture of the 4-substituted and 2-substituted products. Via silica gel chromatography, the 4-substituted product is obtained purely, after which it is reacted with amine **C** to produce the desired novel derivatives of **1**. The inhibitory activity of the new compounds towards Rac1 is determined and compared with the inhibitory activity of **1**. The most active compounds were further investigated for their activity towards cell migration.

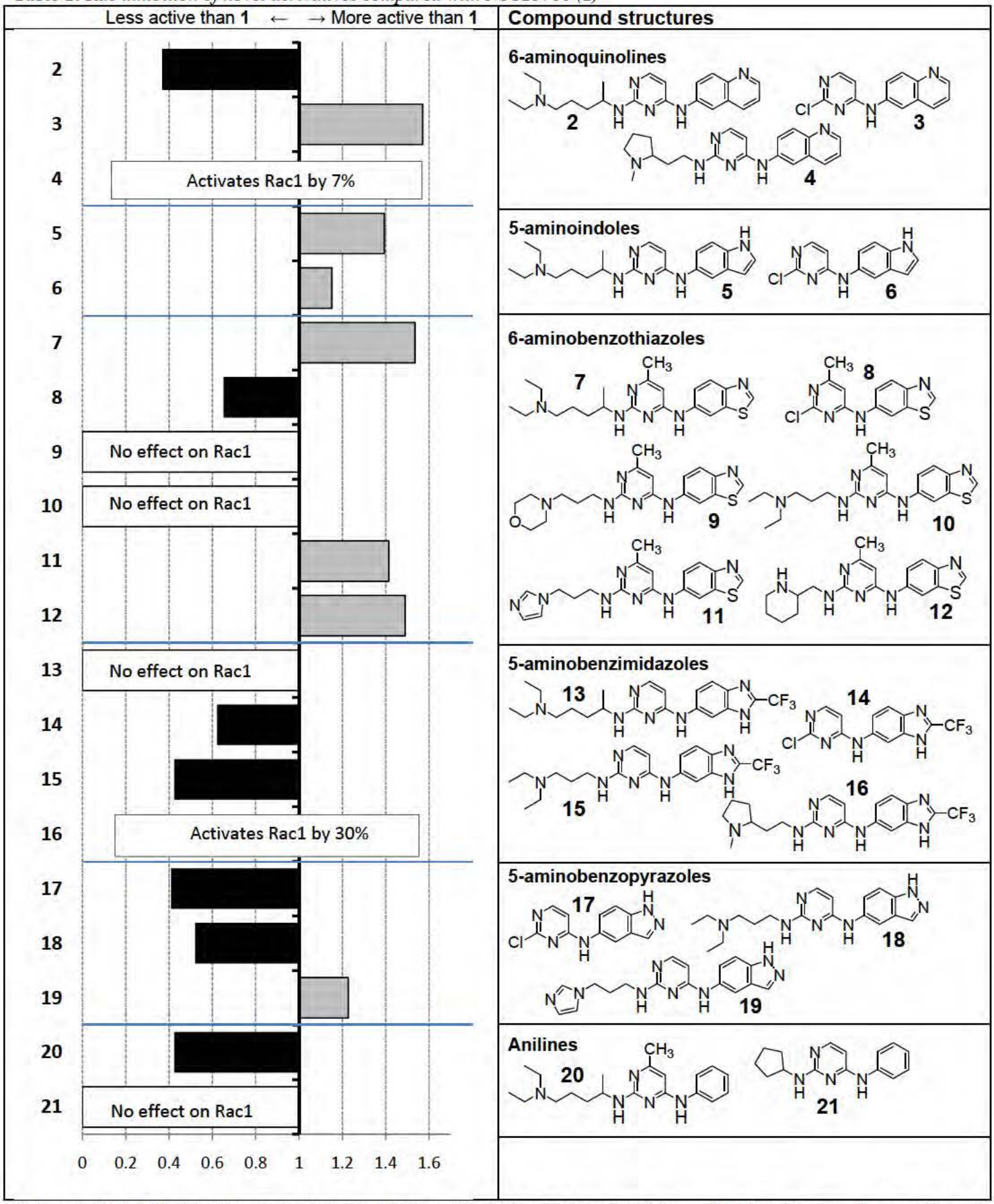
Scheme 1: *Synthetic scheme for the preparation of analogues of NSC23766*



Initial screening
All new compounds that were synthesized were tested for their Rac1 inhibitory activity in MDA-MB-435 metastatic breast cancer cells via the Rac1 pull-down assay procedure. After incubation for 24 hours at a concentration of 50 µM, seven of the twenty compounds were shown to be more active inhibitors than NSC23766 (compound **1**) in the screening for Rac1 inhibitory activity (Table 1). Seven other compounds from the library also showed inhibition of Rac1, but to a lesser extent than NSC23766, while four compounds did not have any significant effect at all. Finally, two of the compounds appeared to be activators of Rac1. When analyzing the compounds grouped by the arylamino building blocks **B**, the following can be remarked: Of all compounds, the 6-aminoquinoline derivate **2** is structurally most related to compound **1**, since it has both an identical amino-substituted aliphatic group **C** as well as a quinoline group in building block **B** as in **1**. Nevertheless, this compound appears to be a much less active Rac1 inhibitor than **1**. In contrast, compound **3**, which is lacking the amino-substituted aliphatic group **C** and instead has a chloro-substituent, appears to be one of the most active inhibitors in this set of compounds. This result, combined with observations in the other compound groups, indicates that the presence of an amino-substituted aliphatic group **C** is not a strict requirement for inhibitory activity. For compound **4**, a very modest activation of Rac1 is observed. The 5-aminoindole derivatives **5** and **6** both are Rac1 inhibitors that are somewhat more active than compound **1**, whether an amino-substituted aliphatic side chain **C** is present (**5**) or not (**6**). Of the six 6-aminobenzothiazole derivatives, three (**7**, **11** and **12**) were demonstrated to belong to the group of compounds that inhibit Rac1 to the largest extent of the currently available compound library. Interestingly, the other three compounds are less active (**8**) than NSC23766, or not significantly active at all (**9** and **10**). Of the four 5-aminobenzimidazole derivatives, three (**13**, **14** and **15**) are much less active than **1**, while compound **16** appears to activate Rac1 with 30% compared with vehicle. The 5-aminobenzopyrazole derivatives are either much less active Rac1 inhibitors

(**17**, **18**) or only slightly more active inhibitors (**19**) than NSC23766. The anilines **20** and **21** are both much less potent Rac1 inhibitors than compound **1**. From the above results, it was decided to further investigate the active compounds from the 6-aminobenzothiazole class.

Table 1: *Rac inhibition of novel derivatives compared with NSC23766 (1)*



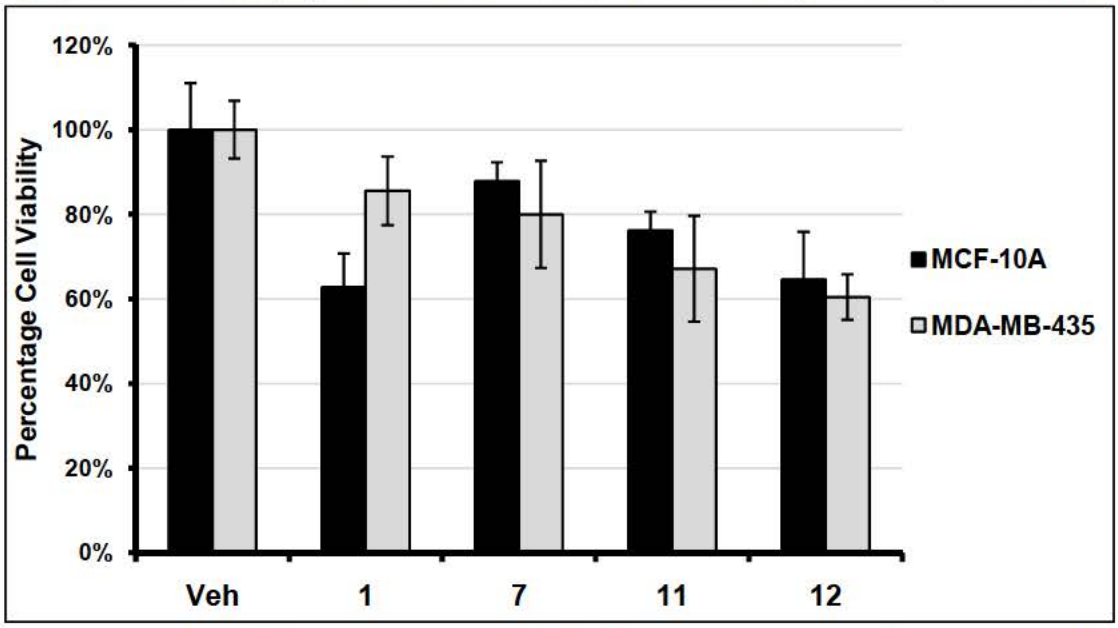
Quiescent MDA-MB-435 cells were treated with vehicle, or 50 μM Rac Inhibitor for 24 hrs, and total protein extracts were obtained. Rac activity was measured using G-LISA Rac1 Activation Assay (Cytoskeleton, Inc.). Average chemiluminescence of each

experimental sample, was measured from a GTP-Rac affinity plate. Results are shown as the fold activity compared to Rac1 inhibition by the parent compound NSC-23766 (19.7% inhibition at 50μM).

Effects of NSC23766 derivatives on cell viability

In order to establish whether the active aminobenzothiazoles **7**, **11** and **12**, as well as compound **1** demonstrated general toxicity, the cell viability of both the epithelial cell line MCF-10A and the highly metastatic breast cancer cell line MDA-MB-435 in the presence of these compounds were determined. In these assays, both cell lines were exposed to 50μM of the Rac1 inhibitors for 24 hours. As can be seen in figure 3, the observed cell viability with compounds **7** and **11** is comparable to NSC23766 (**1**) and does not demonstrate substantial toxicity to either cell lines, although compound **11** seems to be somewhat more toxic to the MDA-MB-435 cell line than the other compounds. While relatively comparable, compound **12** appeared to have the greatest effect on cell viability in both cell lines, and was not further studied.

Figure 3: *Cell viability of MCF-10A and MDA-MB-435 in the presence of* ***1****,* ***7****,* ***11*** *and* ***12***

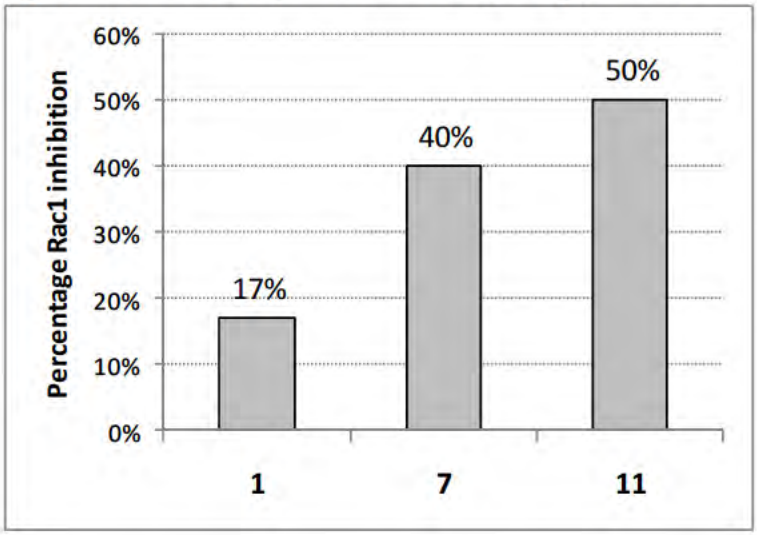


Quiescent MDA-MB-435 breast cancer cells or MCF-10A mammary epithelial cells were treated with vehicle (DMSO), or 50 μM **1**, **7**, **11** and **12** for 24 hrs. Cell viability was measured using a MTT-Cell survival and proliferation kit (Millipore, Inc.). Average Abs. at 600nm of each experimental sample (N=3), was measured from a 96 well plate on a Microplate Reader (Bio-Rad). The mean values ± SEM are presented relative to vehicle (100%).

Effect of selected NSC23766 derivatives on Rac activity

Before further investigating the effects of the compounds of the 6-aminobenzothiazole class for their effects on cell migration and adhesion, we initially carried out a confirmatory assay of the Rac1 inhibitory activity of compounds **1**, **7** and **11**. A 24 h incubation of MDA-MB-435 cells with 50 μM NSC23766 resulted in only a 17% inhibition of Rac1 activity. As can be seen in figure 4, compounds **7** and **11** gave 40% and 50% inhibition of Rac1 activity respectively under the same conditions. Therefore, this experiment confirms that NSC-23766 derivatives **7** and **11** are more efficient Rac1 inhibitors than the parent compound in the highly invasive cancer cell line MDA-MB-435.

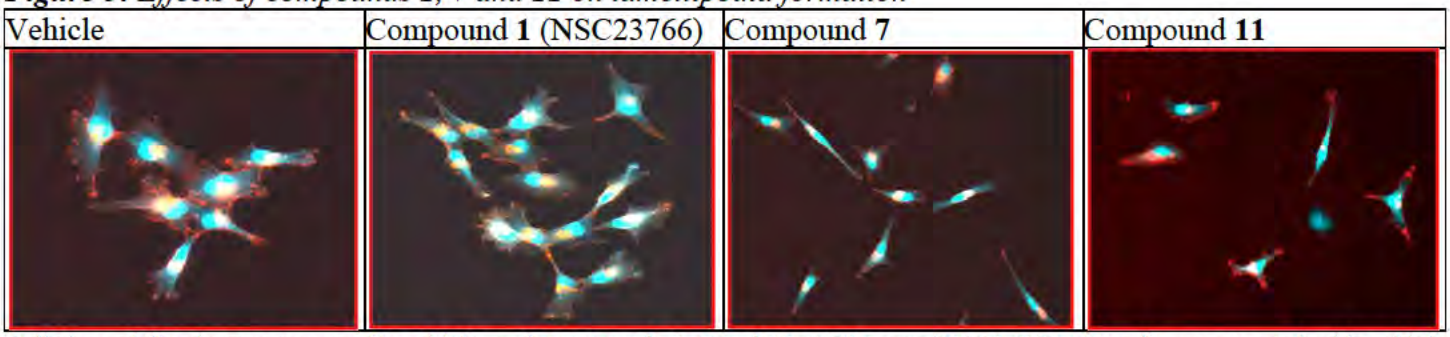
Figure 4: *Inhibition of Rac1 activity by compounds **1**, 7 and **11***



Effects of selected compounds on cell shape and cell migration

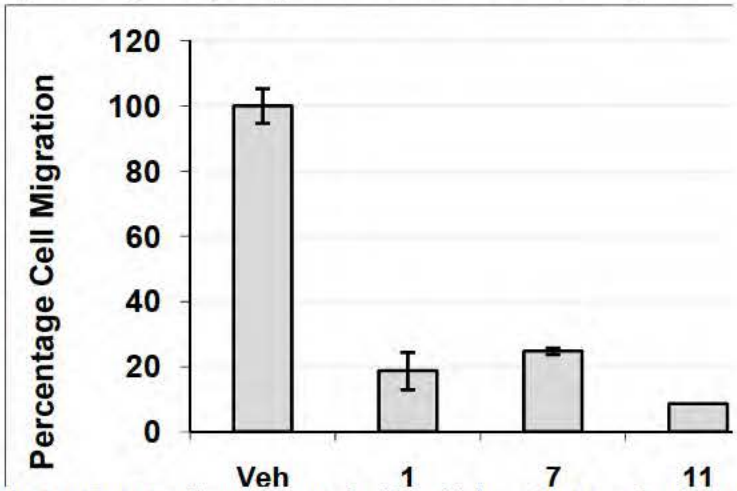
We next tested the effect of compounds **1**, **7** and **11** on cell shape, extension of cell surface actin structures called lamellipodia, and directed cell migration that are hallmarks of Rac-regulated cell functions. MDA-MB-435 cells were treated with vehicle or 50 µM **1**, **7** and **11** for 24 h. From figure 5 it can be observed that treatment of the cells with NSC23766 does not present a significant difference compared with vehicle. In contrast, compounds **7** and **11** are clearly much more efficient than NSC-23766 at reducing cell spreading and extension of lamellipodia, and directed migration towards serum. The effect of compounds **7** and **11** on cell migration was determined, and is represented in figure 6. Interestingly, although at 50 µM concentrations these compounds inhibit Rac1 activity only by 40-50%, lamellipodia formation and cell migration is reduced by 80-90%. It is possible that *in vivo*, a 50% inhibition of Rac1 activity may be sufficient to exhibit the observed effects. Alternatively, **7** and **11** may have non-specific effects on other signaling molecules that regulate cell migration such as Cdc42 and Rho GTPases.

Figure 5: *Effects of compounds **1**, 7 and **11** on lamellipodia formation*



Cell shape following treatment with vehicle, and compounds **1,** 7 and **11**. GFP-tagged MDA-MB-435 cells were treated with vehicle or 50 µM compounds for 24 h, fixed and stained with rhodamine phalloidin to visualize the actin cytoskeleton.

Figure 6: *Effects of compounds* ***1****,* ***7*** *and* ***11*** *on cell migration*

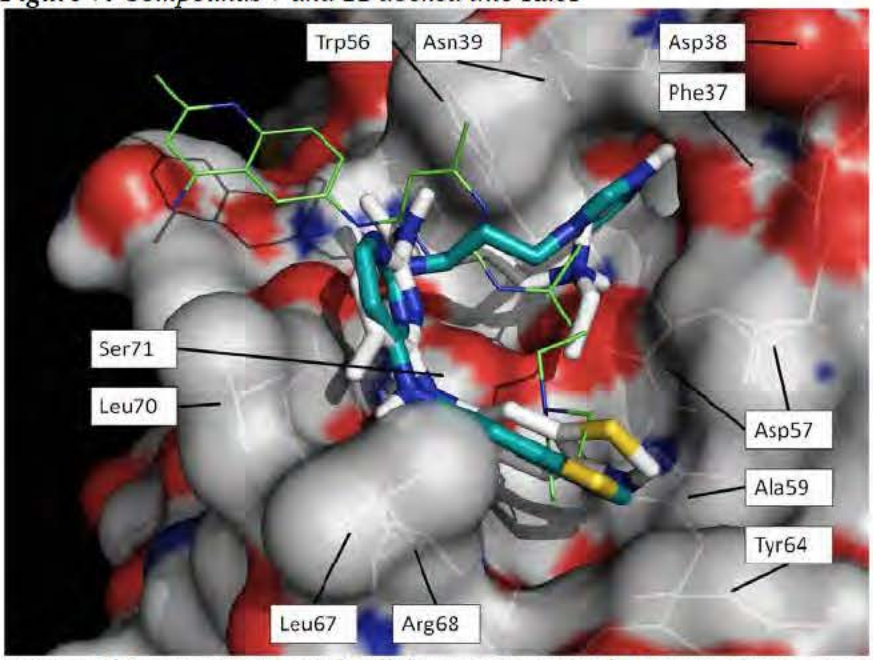


MDA-MB-435 cells were treated with vehicle or 50μM each of compounds **1**, 7 and **11** for 24h. Equal numbers of cells were placed on the top well of Transwell chambers that contain a membrane with 8μ diameter pores. The bottom well contained serum. The number of cells that migrated to the underside of the membrane was quantified for each treatment. Results are shown relative to vehicle (100%).

Docking of 7 and 11 in the crystal structure of Rac1

The crystal structure of Rac1 with the inhibitor NSC23766 was recently revealed in a patent application (20). This structure has been used in a virtual screening of the ZINC database, and five new compounds with different core structures were identified as Rac1 inhibitors with IC_{50}s from 12.2-57.2μM (21). Utilizing Autodock 4 (22), we now docked our novel Rac1 inhibitors **7** and **11** in this crystal structure. Similar as the compounds identified from the ZINC database, both compounds **7** and **11** bind in multiple conformations of close energy within a binding pocket formed by amino acids Phe37, Asp38, Asn39, Trp56, Asp57, Thr58, Ala59, Tyr64, Leu67, Arg68, Leu70 and Ser71. The lowest energy conformation of the most populated cluster of the docking results of both compounds is represented in figure 7, together with the position of compound **1** (green), in the crystal structure. Although NSC23766 (**1**) is stretched over the surface of Rac1, compounds **7** (light grey) and **11** (cyan), in all lowest energy conformations, including the ones shown, assume a bent shape and seem to dock deeper into the binding pocket. We will utilize docking in order to guide the design of inhibitors that will bind more tightly with Rac1.

Figure 7: *Compounds 7 and* ***11*** *docked into Rac1*



Compound **1**: green; Compound 7: light grey; Compound **11**: cyan. Figure generated with PyMol

Conclusion

Via minor structural modifications of NSC23766 we have identified two compounds (**7** and **11**) that are ~2-3 times more active as inhibitors of Rac1, while presenting minimal toxicity to epithelial cells. Both compounds were shown to have a much more dramatic effect on lamellipodia formation and cell migration than the lead compound NSC23766. Whether this is related to non-specific effects remains to be determined. Nevertheless, our experiments have demonstrated that the search for novel and more potent inhibitors of Rac1 can lead to compounds that are very effective in interfering with a key biological process related to metastasis. We expect that, utilizing molecular docking as an aid in the design, other compounds with increased efficacy can be synthesized and tested. Potentially, this could lead to a novel pharmaceutical treatment that prevents the spread of cancers to other tissues.

Acknowledgements

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Resumen

Trasfondo: La familia de Rho GTPasas son interruptores moleculares que controlan las vías de señalización y regulan una cantidad de funciones celulares. Rac1, un miembro de la familia de Rho, desempeña un papel crítico en varios aspectos de progresión del cáncer y metástasis. Rac no está mutada en cánceres humanos invasivos, pero se encuentra sobreexpresada. Debido a que las Rho GTPasas son activadas por factores de intercambio de nucleótido guanina (GEFs), inhibir la interacción de Rac con sus GEFs es una estrategia específica para el bloqueo de la activación de Rac.

Métodos: El IC_{50} de NSC23766, un inhibidor específico de Rac1 con un subconjunto de GEFs, es demasiado alto para uso terapéutico, por lo que inhibidores más eficaces son necesarios. Por lo tanto, iniciamos la síntesis de nuevos derivados del NSC23766 con modificaciones en los sustituyentes conectados al anillo central pirimidina, y examinamos su actividad inhibitoria contra Rac1.

Resultados: Varios derivados de NSC23766 inhibieron la actividad de Rac1 en células cancerosas con mayor eficiencia que NSC23766. Los nuevos compuestos no son tóxicos para las células epiteliales mamarias normales, pero son más eficientes que NSC23766 en la inhibición de migración celular y reducción de propagación y extensión de lamelipodia, funciones celulares controladas por Rac1 que contribuyen a la invasión de células cancerosas.

Conclusiones: Basado en los resultados, concluimos que los nuevos compuestos muestran en avance un potencial de desarrollo como inhibidores de metástasis de cáncer de seno.

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